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Microscopy. II. Electron Microscopy: *A Review*

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INTRODUCTION

History and theory.—Part I of this review (172) has discussed the various methods which have been designed to utilize all the properties of light waves in the optical spectrum for the purpose of analyzing the structure, the molecular orientation, the refractive index, the anhydrous organic mass, the chemical composition, and the thickness of a biological specimen. For two-dimensional structural analysis, it was shown that, as predicted by Abbe's Rule, it is impossible to extend the resolving power of an optical microscope below one-half the wave-length of the illuminating light so that 0.1 μ is the limiting resolution afforded by any optical microscope.

Although x-rays were demonstrated in 1912 to have wave-lengths of the order of a few Angstrom units and possess good penetrability, it was found to be impossible to use these radiations in a microscope system, because of the lack of suitable refractive materials to focus the beam. Instead, it was found necessary to develop methods of x-ray diffraction analysis which enable the operator to perform the operations of a lens mathematically and to derive expressions for the atomic density of simple specimens. But no progress was made in directly visualizing structure below 0.1 μ until the wave properties of electrons were revealed. De Broglie (1924) postulated that electrons exhibit a periodic behavior and that the wave-length corresponding to this periodicity must obey the law $\lambda = (150/V)^{1/2}$.[1] Two years later, Busch (54) showed that magnetic or electrostatic fields possessing rotational symmetry act as true lenses for an electron beam travelling approximately along their axis of symmetry. Incorporating Schrödinger's theories with these two principles, physicists found it possible to design a microscope with a beam of electrons as an illuminating system and with magnetic or electrostatic fields as lenses (152). From this time, development of a high-resolving-power electron microscope was relatively rapid. In 1935 the first electronic image having a resolving power greater than that obtainable with the light microscope was obtained in Germany (86). By 1940 commercial instruments, with limiting resolving powers of 20–25 A were being manufactured in Germany and America, and by 1946 improvements in technics and design had made it possible to demonstrate resolving powers of 8.5–15 A (8, 128).

Such resolutions are made possible by the fact that electrons moving down a potential gradient of 60 KV, for example, will have an associated wave-length of 0.0488 A, or about 100,000 times shorter than the wave-length of visible radiation. The effective numerical aperture of an electron microscope is extremely low, because of the fact that electro-magnetic lenses cannot be corrected for spherical and chromatic aberration, so that the finest resolution actually available under ideal conditions is about 8–10 A (128).

There are a variety of excellent short reviews (165, 274) and longer treatises (65, 101, 275) discussing the electron-optical theory of the electron microscope and the various designs of electrostatic and electro-magnetic instruments (116) now commercially available, to which the reader is referred for detailed discussion of these subjects.

METHODS

Electron-optical theory shows that it is possible to build electron-optical counterparts of most optical instruments. The electron microscope is in the-

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[1] λ is the wave-length in Angstrom units (10^{-8} cm.) and V the potential in volts used to accelerate the electrons.

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ory, therefore, a rather exact counterpart of the light microscope. Because of the necessity of using high accelerating potentials in the production of the electron beam and of handling the beam entirely in a vacuum, its physical form is completely different, and it imposes quite different requirements for specimen preparation.

Specimen requirements.—All specimens must be placed in the vacuum under which the instrument is operated so that there is, as yet, no possibility of observing wet or living specimens. Specimens must also be so thin as not to show appreciable electron absorption because image contrast arises primarily from the absence of electrons scattered elastically out of the direction of the beam, rather than from the absence of those absorbed in the specimen. Electron absorption must also be avoided because kinetic energy lost to the specimen causes damaging heating and rupture. Since electrons of only one velocity are used, variations of electron scattering and absorption power of the object points due to variations in atomic densities are the primary cause of variations of intensity at the image. Thus image contrast in the electron microscope arises primarily from variations in atomic densities. It has, however, not yet been possible to determine a simple relation between the mass density of the object point and the intensity observed in the corresponding image point, so that it is not yet possible to quantitate intensity variations in the electron image to mass or density variations in the specimen.

These conditions, applied specifically to biological systems, impose the following requirements for specimen preparation:

(*a*) Specimens must be thoroughly dehydrated and/or fixed prior to examination. (A further "fixation" arises after exposure to the electron beam [135].)

(*b*) Adequate contrasts of atomic density must be present or be induced in the specimen. Most materials of biological interest consist predominantly of the elements C, N, and O which have nearly the same atomic weight. Thus variations in atomic density are primarily variations in local concentrations of these elements which are, in turn, related to the distribution of water originally present in the fresh material. Artificial image contrast may be introduced by staining or fixing with such electron-dense compounds as osmium tetroxide, phosphotungstic acid, and phosphomolybdic acid. (Recent evidence [194, 198] indicates that osmic acid does not appreciably affect the electron density of proteins that it fixes.) Contrast may also be enhanced by the use of appropriate lenses (131, 134), apertures (134), and by metal shadowing (261, 262). Care must also be taken to mount specimens on a sufficiently thin supporting film so that density variations in the specimen are not obscured by the constant density and thickness of the supporting film.

(*c*) Specimens must be no thicker than 0.2 μ if resolutions less than 0.1 μ are desired and even thinner for optimum resolutions. The size, over-all shape and silhouette of thicker objects can, of course, be studied. To satisfy these criteria it has been found necessary to prepare biological tissues either by isolating cell components, by selecting thinly spread portions of cultured or smeared cells, by sectioning fixed and embedded tissues, or by making replicas ("finger-prints") of the surface of thick specimens.

Specimen preparation.—Isolated cell components: The way was first opened for the study of intact cell components when it was shown (21, 60) that mitochondria and microsomes could be successfully isolated by means of differential centrifugation. Methods were then devised for isolating interphase chromosomes (64, 174). Such isolates can then be easily prepared for electron microscopy. The morphology of the components so isolated depends both upon the electrolytic and osmotic properties of the suspending solution, and upon the mechanical methods used to fragment and dissociate the original tissue.

Tissue culture and cell smears: The first successful electron micrographs of a complete cell were published as the result of a method, introduced by Porter, Claude, and Fullam (210), for the examination of cells in tissue culture. The method takes advantage of the fact that cultured cells have a tendency to flatten out at the periphery so that areas in such spread cells can be found which are sufficiently thin for electron microscopic examination. The technic consists in growing cells upon a plastic film and, after fixation in vapor or drops of the desired fixative, transferring the cells together with the film to which they adhere to the supporting screen of the electron microscope. A modified version of the original method has now been published in useful detail (164).

Preparing cells in this manner, it became possible to examine cellular structure, at electron microscopic magnifications, under the influence of various fixatives (210, 213). All fixatives tried, except osmic acid, caused a gross contraction during drying which was accompanied by striking submicroscopic distortions. After formalin fixation no mitochondria are visible, whereas after fixation in fumes of osmic acid for periods from 5 minutes to 48 hours the mitochondria are observed as particulate bodies clearly demarcated from the surrounding cytoplasm. The authors' conclusion that fixation in osmic acid most closely preserves the living cell structure has been borne out by all following investigators (14, 48, 196, 197).

Bessis and Bessis and Bricka (29, 30, 31) first studied thinly-spread portions of blood cells which were simply spread upon a film-coated coverslip and then transferred, with their supporting film, to specimen grids. They observed predominantly air-dried or alcohol-fixed red and white blood cells which all showed, after this treatment, an identical gross granular structure. Bernhard, Braunsteiner *et al.* (23, 25, 40) improved upon the method by introducing brief fixation in osmic acid vapors, similar to that performed upon tissue cultures, so that comparably fine structure is revealed. Smears were also made at 37° C. to permit more complete spreading, and unspread cells were washed off. Simple smear preparations have also been used most successfully for the study of hemolyzed red blood

cells (31, 158). Unhemolyzed or untreated cells are too electron-opaque for study of anything but their silhouette (157, 219).

Thin sectioning: The size and shape of cytoplasmic components are successfully studied in smeared and cultured cells but the nucleus and perinuclear region are too electron-opaque for observation. To study these regions and to evaluate the quantity of any component per cell it has been necessary to develop methods to cut 0.05–0.20 μ thick sections from whole tissue. Since the earliest attempts of v. Ardenne (7) and Sjostrand (247) to cut thin sections with ordinary microtomes, many adaptations of commercially available microtomes have been built, and many new designs described.

The direct screw-feed of the rotary Minot microtome was adapted to advance one-quarter the normal value (224) and later 0.05 micron per tooth (105). Pease and Baker (204) changed the wedge angle of the Spencer microtome "820" to achieve a transmission one-tenth that of the calibrated value (i.e., 0.1 μ). Hillier and Gettner (111, 134) improved upon this system by adding a motor drive with vibration-free coupling, mechanical improvements in the advance mechanism, and a water trough to float the sections off the knife. This method has been used successfully in a number of laboratories (37, 122, 245, 256) and was the first to render resolutions of 200–300 A (134) possible. A thermal expansion method was then introduced (187) which involved replacing the advance mechanism of a Spencer microtome with one that advances by thermal expansion of a previously cooled brass block. Fernandez-Moran (97) achieved thermal expansion advance by embedding frozen objects in gelatin, and mounting these in a Spencer microtome which thereupon advanced by expansion of the ice block.

The earliest microtome built especially for electron microscopy was based upon the principle that (193) the thickness of sections may be determined by the rate at which the object moves and the speed of a rotating blade. Fullam and Gessler (100) constructed two different high-speed microtomes to which Claude (62) added the necessary adjunct of a liquid reservoir. This microtome was used in a number of early studies (60, 62, 107–109), but has now been abandoned in favor of recent simpler and more effective designs. Bretschneider (43, 45) furthered the development of the "rocking" microtome, which has been used most successfully by the European workers. In this microtome the displacement of the object toward the knife is reduced by means of a lever with arms of different lengths. The initial design was followed up by Danon and Kellenberger (73) who constructed a rocking microtome in which, because of the wedge-shaped construction of the part moving the lever, a further reduction of the distance between the blade and the object was obtained.

It has recently been found to be extremely important, for the sectioning of plastic-embedded tissue, to avoid contact between the block and the cutting edge during a noncutting stroke. Methacrylate undergoes a slight expansion after the cutting blade has passed through it, so that, if the block touches the knife on the up-stroke, undesirable wetting and dulling of the blade occur. Therefore, most workers are now concentrating on new microtome designs which avoid block and knife contact on a noncutting stroke. This was first achieved in an early design by Claude and Blum (60) in which the specimen advances continuously by rotating in front of a stationary knife. By adding a motor drive, glass cutting edge (see below), and trough Palade (197, 198) found this system excellent for cutting plastic-embedded sections 0.06 to 0.1 μ thick. Similar revolution of the specimen in front of a stationary hand-sharpened steel knife (133) or razor blade (250) was combined with thermal expansion of a brass rod in two successful and easily-built microtomes (133, 250) developed recently. The advance of both microtomes is calibrated while that of Sjostrand's has produced sections in which a resolution of 25 A can be demonstrated (Fig. 5). Another design (114) involving a direct screw feed, adds two rachets so that the specimen is retracted one unit at the end of a stroke for every two units of advance. Still another advances the specimen by a rotating inclined plane bearing against a traveling nut and facilitates cutting alternate 0.1 and 1.0 μ sections (110).

Thus there are at present available a variety of simple microtomes providing advances of from 0.05 to 0.2 μ of the specimen block to the cutting edge. Few of these designs are commercially available but most are easily built. The principles of design which have been found to be indispensable are motor-driving, regular calibrated advance, a fluid reservoir for the production of serial sections, and contact between the block and the knife solely during the cutting stroke. These are incorporated into the newest designs (257).

Once the desired mechanical advance has been achieved the quality of the embedding material and the cutting edge become the limiting factors in the achievement of satisfactory sections. Paraffin embedding was used by the early workers, but the paraffin had to be removed from the sections prior to examination because they were not thin enough (62, 107, 108, 109). Embedding media, such as camphor and naphthalene, which could be removed by volatilization under vacuum, were not more successful (108, 109, 134). Ester wax (26, 43) has been used in Europe. Double embedding first in 10 per cent celloidin and then in paraffin was introduced by Pease and Baker (204) and found to produce suitably hard blocks of good cutting quality. Triple embedding (202) was introduced to provide even harder blocks for such tissues as hair, teeth, and skin. Hillier and Gettner discussed the merits of double embedding (134) and concluded that removal of the embedding introduces gross movement of tissue structures and should be avoided. Much of the paraffin is actually removed, without distortion, by sublimation under the beam. This is equally true for all embeddings, since the finest resolutions (197, 198, 250) have consistently been demonstrated in sections from which the embedding has not been removed. Artifacts introduced by the sectioning and mounting, such as compression and knife marks, are easily recognized deformations which need not influence the interpretation of the structures. A method for sectioning hydrocarbon gels has been described (12).

A plastic embedding, now in most general use, was introduced by Newman *et al.* (187). Tissues are immersed in liquid *n*-butyl methacrylate which, after incubation at 45° C. with a catalyst or under ultraviolet light (167), polymerizes and fixes the tissues in an extremely hard, colorless resin. The hardness of the embedding may be controlled by the amount of catalyst used or by the addition of methyl methacrylate. This embedding has the advantage of being as hard as desired and thus eliminates the great compression (about 30 per cent) inevitable in sections of celloidin-paraffin embedded material. Its disadvantages are introducing occasional gross injuries to tissue during polymerization, needing a supporting film under the election beam, and undergoing expansion during sectioning. However, the thinnest sections and, therefore, the finest resolutions have been achieved using this embedding (250, 251).

As a result of the increased magnification with which tissue sections are observed, increasingly smaller knife defects become evident and destructive of sections. To improve upon the quality of a steel knife (133) or razor (250) edge, it was found necessary to replace stropping of the blade with careful hand sharpening on glass plates with fine abrasive powder. Excellent edges may be produced by careful hand sharpening, but many laboratories find this procedure too tedious and unsuccessful, or find stropping quite satisfactory (37). Thus the introduction of a glass knife (159), whose edge is superior to a commercially sharpened steel knife, has been a most useful contribution, since its low cost and simplicity make it easily available to all.

Fixation: Finally, when a satisfactory 0.05–0.2 μ section has been obtained, interpretation of the image revealed therein depends upon all the factors concerned in preparing the tissue itself. The effects of coagulation and distortion of tissue protein and shrinkage of cells produced by the known methods of fixation become very marked and objectionable under the higher resolution of the electron microscope. It was soon established (44, 60, 108, 204, 210) that osmic acid, as the finest protein precipitant, best preserves living cell morphology at the highest magnifications. Its disadvantage is poor penetrability which may be counteracted by preliminary formalin fixation followed by osmic acid with potassium dichromate (48, 108). However, fixation by perfusion or in free cell suspensions has proved to be a better way to circumvent the poor penetrability. Ornstein *et al.* (196) demonstrated the value of formalin fixation prior to osmic acid while others suggested a combination of osmic acid with lanthanum salts (67). Formalin used alone is useful for nuclear studies (46, 232, 233). Attempts to section unembedded frozen-dried material (98) have been unprofitable because of the necessity of using liquid spreading media in transferring thin sections to a specimen grid. The value of embedded frozen sections was recently demonstrated by Sjostrand (250) but is believed by others (49) to introduce unwelcome artifacts.

Palade (197) determined that, in whole tissue blocks, osmium fixation is preceded by an acidification of the tissue, which considerably alters cell morphology. The quality of fixation is therefore highly dependent upon the pH of the fixative, and it is optimal when the fixing solution is buffered at pH 7.3–7.5. Fixation above or below this pH produces thick nuclear and cell membranes, cytoplasmic vacuolization, nuclear granularity, and distortion of the intestinal brush border. The electron micrographs (197, 198, 250, 251) demonstrate that this fixation is the finest obtained to date and that many observations upon cellular structure have been reported of fixation-induced artifacts. However, there is still a great need of alternative fixatives to point out properties of cell components other than osmiophilia. Electron-staining with phosphotungstic or phosphomolybdic acid has, so far, been used exclusively on fragmented tissue (20, 239) while additional stains are now being suggested (156).

Three-dimensional imaging: This discussion has so far concerned only the achievement of two-dimensional images. Three-dimensional imaging can be obtained by using either stereoscopic-viewing or metal-shadowing technics. By using a special specimen holder, available on commercial machines, for tilting the specimen at opposite angles to the beam for successive photographs, paired images are obtained which, when viewed through a stereoscopic viewer, give an excellent three-dimensional image. The full merit of this technic has awaited methods by Anderson (2, 3) and by Williams (260) for preparing specimens in such a manner that all compression and surface tension artifacts due to drying are eliminated and the dehydrated specimen retains its true three-dimensional orientation in the vacuum.

The method which is now standard for determination of an approximate three-dimensional image of any specimen is that of metal-casting introduced by Williams and associates (261, 262). Suitable metals such as silver, chromium, gold, palladium, platinum, etc. are evaporated upon a specimen at a small angle so that a thin film of metal covers the whole specimen except for a "shadow" when the specimen itself impedes the passage of the metal. From the shape and dimension of this shadow, knowing the angle at which the metal was spattered, the height and contour of the specimen object can be inferred. Thorough discussions of criteria for good shadowing and artifacts which may arise from too heavy a shadow or too grainy a metal are given by Williams and Backus (261).

A useful method for determining structure not visible in a transmitted image is that of examining a surface replica ("finger-print") of the specimen. The various types of replicas which can be obtained include metal or plastic replicas, pre-shadowed replicas, and pseudo-replicas (87, 120, 261, 268). Although extremely useful, application of this technic to biological problems has been limited to date (60, 158, 199, 223).

Only methods useful for preparation of whole cells or cell components have been included here so that for more detail and methods for preparation of viruses and particle suspensions the reader is referred to the many excellent reviews concerning specimen preparation (65, 87, 268) and general biological applications (48, 65, 130, 268) which are available. Several comprehensive bibliographies are also available (66, 166, 188). The many conditions and requirements for adequate manipulations of the microscope (i.e., maintenance, alignment, aberrations in the image, etc.) also require special treatment, but it is sufficient to say here that all the prerequisites for adequate cleanliness, and manipulation of any optical microscope are paralleled and very greatly magnified in the case of the electron microscope.

BIOLOGICAL APPLICATIONS

Isolated cell components.—Claude and Fullam (61) examined preparations of the mitochondrial fractions isolated from rat lymphosarcoma cells and found the first optical evidence for the existence of a mitochondrial membrane. Formalin-fixed mitochondria became relatively transparent to the electron beam after standing in the ice-box for several days so that an enclosing membrane was seen together with 80–100 mμ elements within the mitochondrion. Dalton *et al.* (70) revealed mitochondrial membranes by isolating the mitochondrial fraction of normal hepatic cells, spontaneous and induced hepatoma, and the S-91 melanoma of the mouse in various concentrations and combinations of electrolytes and nonelectrolytes so that, under these conditions, the membranes tore or the contents lysed. Relatively large particles (60–100 mμ) appeared in mitochondria isolated in physiological saline, while a considerably smaller granular substance was found in those isolated in 0.88 M sucrose. Of particular interest is these authors' observation of particles in the mitochondrial fraction smaller than those visible to the light microscope. Muhlethaler *et al.* (185) measured the membrane of metal-shadowed mitochondria lysed in distilled water and estimated it to be about 20 mμ thick. Recently the mitochondrial fraction of rat liver mitochondria has been separated into two morphologically and biochemically distinct fractions (153). The smaller fraction, consisting of rodlets with length and width about one-half of those for the rods in the other fraction, has the

high pentosenucleic acid ratio which has been considered characteristic of the "microsome" fraction.

The only electron microscope study that has been made to date upon the small particle or "microsome" fraction (253) reveals the existence of at least two discrete components in this fraction of mouse liver and hepatoma. One consists of rather poorly absorbing globular structures, often appearing in chains and with diameters of about 100 mμ. The other consists of very electron-dense spheres with diameters of 20 mμ.

Chromosomes have proved to be difficult to study with the electron microscope because of difficulties of isolation and because they are large enough to be quite opaque to the electron beam. The many early electron microscopic studies of chromosomes were primarily to evaluate the merits of various methods of preparation and do not demonstrate any finer resolution than available in light micrographs. Elvers (90) reviewed the early work and presented a comprehensive study of various treatments, particularly of enzymatic digestion, on sectioned and micro-dissected pachytene chromosomes of Lilium. Buchholz (53) discussed preparative methods and studied microdissected chromosomes of *Zea mays*. The giant chromosomes of Drosophila have been studied in thin sections (37, 206), by dissection (90), by smearing technics (126, 241), by parlodion replica (199), and by the use of shadowed formvar replicas (272). Most investigators agree that the chromosomal bands are made up of small granules of approximately equal size. A network of filaments appears between these bands which Palay and Claude (199) reported to be digested by desoxyribonuclease (DRNase) and are thus an artifact produced by stretching the chromosomes. However, Yasuzumi *et al.* (272) observed these interband fibrils, with a periodicity between 30 and 60 mμ, in all specimens prepared either by direct or replica technics and even after DRNase digestion and suggest that these fibrils correspond to the chromonemata. Others consider the fibrils are too irregular in size and alignment to be chromonemata (126, 206, 241).

The introduction of methods for isolating chromatin threads from the resting nuclei (64, 174) was immediately followed by electron-microscopic examination of such preparations. Hovanitz (141) examined threads isolated by the Waring Blendor method from chicken erythrocyte nuclei, and found that the procedure of Mirsky and Ris (175) for the preparation of "residual chromosomes" by treatment of the threads in molar sodium chloride renders the threads less opaque and hence more easily studied. A spiral structure, similar to that observed in pachytene chromosomes of Lilium (90) was seen in untreated whole threads, while "residual chromosomes" possessed a beaded structure. A later study (142) presented models of superimposed helices for the structure of the whole chromosomes and showed that the fragments following DRNase digestion and NaCl dissolution were also helical. Similar twisted or helical filaments appeared after still further NaCl dissolution (75). Hoffman-Berling and Kausche (137) reviewed available electron-microscopic evidence concerning chromosome morphology and reported that typical interphase chromosomes isolated from chicken erythrocytes are polytene. They show a fibrillar composition organized into two helices wound around each other with occasional evidence of striation in the fibrils.

Yasuzumi and co-workers (270, 271) have examined threads isolated from carp and tortoise erythrocyte nuclei and from human leukocyte nuclei. They observed double-stranded helices that were Feulgen-negative with small granules of Feulgen-positive material attached and consider that the coiled threads represent chromonemata, while the DRNase is localized in the granules. A few of the published micrographs show an unusually regular structure and are so dissimilar to other photographs of the threads published by themselves and by other authors as to suggest, in these cases, a possible bacterial contaminant (139). Calvet *et al.* (56) broke calf thymus lymphocytes by vacuum rupture and obtained meshes of fibers which they interpret as the classical chromatin net, rather than as chromosomes.

The interpretation of these accumulated studies upon isolated chromatin threads has recently been challenged by Lamb (154, 155). As the result of optical, phase, and electron microscope studies of calf thymus cells after various periods of disruption by sand-grinding, by the Waring Blendor, or by a Potter-Elvehjem type homogenizer, he concluded that the threads are simply complex fragments of drawn-out nuclei. Contrary results were obtained by Mirsky and Ris (176, 226) and Denues (76, 77) who presented extensive evidence to show that the threads actually are "isolated chromosomes." Denues noted that two radically different methods of disruption of chicken erythrocytes (Waring Blendor and Logeman hand colloid mill) produced similar structures at a similar rate, that the threads produced have the same morphological characteristics as the metaphase macrochromosomes of the chicken, and that their fine structure (i.e. doubleness, coiled aspect) is that associated with chromosomes. We may hope that agreement is rapidly reached upon this question of

identity, so that progress may be made in elucidating their important fine structure.

An interesting double membrane structure was first revealed (55) in oocyte nuclei simply spread thinly upon grids. Bretschneider (48) confirmed this observation in sections of leech neuronal nuclei. *Amoeba proteus* also has a double nuclear membrane which Bairati and Lehmann (11) described as composed of two layers, one containing 1200 A pores with 1500 A between centers, while the other is a continuous membrane of densely packed small globular particles. The porous layer is very resistant to mechanical action and to different fixing fluids and contains few lipoids. In sections of *Amoeba proteus* Harris and James (124) described the outer layer as 1000 A thick and the inner porous layer 2000 A thick. Their measurement for the spacing between pore centers is 1200 A, and the pore diameter is 800 A. The situation in amoeba is therefore the opposite from that in the oocytes where the continuous layer is on the inside and porous layer on the outside.

Cell cultures and smears.—Electron-optical studies of cells cultured from a variety of tissues: rat fibroblasts (14) and rat sarcomas (212, 214), mouse (58) and human epithelial tissues (246), mouse (58) and human sarcomas and carcinomas (246), chicken fibroblasts and tumor (63), mouse mammary carcinoma (215), have confirmed the original observations (210, 214) of certain cytoplasmic components, some invisible in optical microscopes, which are common to all cells. The following description of the cytoplasm in cultured cells is consistent for all cell types so far examined (14, 212, 246).

After brief fixation over vapors of osmium tetroxide, mitochondria and lipid granules are readily distinguishable, while the background consists of a scattering of poorly resolved submicroscopic granules, a complexity of dense strands in the endoplasm, and certain intracellular fibrils. After longer treatment with fixative (18–21 hours) and considerable washing, the formed bodies become more sharply defined. They are present in the same form and distribution as before, but much of the embedding matrix of diffuse and frequently fibrous components has been removed (213). There are then three types of formed elements remaining. These are as follows:

(*a*) Fat droplets: These are easily recognized, although their size range overlaps that of other cytoplasmic components, by their great density (due to their high capacity to reduce osmic acid) and by their smooth-edged, usually spherical shape. They are plentiful in cultured cells; large droplets cluster around the nucleus, while submicroscopic ones may appear further toward the periphery of the cell as in Figure 3 (*f*).

(*b*) Mitochondria: These are nearly as osmiophilic as the fat bodies and adopt all the varieties of shapes from filamentous to spherical that have already been amply described as visible in the light microscope. For example, see (*a*) in Figures 1, 2, and 3. They flatten down (dehydrate) considerably during drying since they cast a much shorter shadow than would be expected from an intact cylinder. No clear internal structure can be defined because of the density of the material and confusion with over- and under-lying substances, but an enclosing membrane is evident.

(*c*) Endoplasmic reticulum: A third component, first noted by Porter *et al.* (214) and named later (215) the "endoplasmic reticulum" is present in all cells. It is usually visible only after the longer periods of fixation when it appears as a vesicular or canalicular array of lightly osmiophilic elements, as illustrated at (*b*) in Figures 1 and 3. Its form may vary enormously (i.e. from vacuoles 50–120 mμ in diameter to an unresolved lacy meshwork) in cells within the same culture but is consistent within any single cell, suggesting that the particular form may reflect the state of activity of the cell at the time of fixation. The size range suggests a direct relationship between this component and the small particle or "microsome" fraction of tissues (60, 253). An exact correlation between this component in cultured cells and the endoplasm revealed in tissue sections (see below) is yet to be made.

These three components, embedded in a matrix of variable character, appear consistently in the large amount of material studied, while other inclusions (granules, viruses, fibrils) have appeared only in certain special cell types. Those appearing in cancer cells are of particular interest here.

The first cancer cells examined were those grown from two tumors of viral etiology (63, 215). Cytoplasmic osmiophilic particles of constant shape and diameter, occurring in a regular pseudocrystalline array and thus considered to be "virus-like," were observed in cells grown from both tissue types. Those in the mouse mammary cells were 130 mμ in diameter with a dense 75 mμ center, while those in the chicken tumor I were 67–84 mμ in diameter and those in chicken tumor 10 had a diameter of 60–70 mμ. The relationship of these particles in tumor cells to those observed in ultracentrifuged tumor-inducing extracts of mouse and human mammary cells (112, 117, 200) has not yet been elucidated. No "virus-like" particles were observed in cells cultured from nodes of Hodgkin's Disease (143) or in tissue extracts (138).

Certain osmiophilic particles are observed characteristically in all other tumor cells examined (212, 246), but these are of variable size and morphology and so do not appear to be the same type of viral agent. It is significant that one author (191) reported that no particles of this sort were observed in the Rous sarcoma cells, where the virus particles are prevalent.

This additional cytoplasmic component, which has been called variously a "growth granule" (212), an "ultra-chondrioma" (191) or simply an "osmiophilic granule" (246) was first noted by Porter and Thompson in cells of a rat sarcoma (214). Subsequently, Oberling, Bernhard *et al.* noted similar particles in smear preparations of human leukemic white cells (190), cells of human ascites, and cells of a mouse reticulosis (192) (see below). Porter reported that similar granules could also be seen in actively proliferating rat embryo tissue, and in very rare instances in adult normal cells (212). Their presence in normal white blood cells was then noted by Oberling *et al.* (191), who found the typical granules in 40 per cent of the leukocytes from a normal subject, occasionally in macrophages of inflammatory exudate and of meningial fluid, and rarely in normal monocytes and polymorphonuclear leukocytes. Selby and Berger (246) reported an extensive study of this component in cells cultured from adult and embryonic human epithelium and from human carcinoma, in addition to a survey of various adult and embryonic normal and neoplastic mouse tissues (58). In two cases it was possible to compare the cytoplasm of epithelial-type cells grown from adjacent normal and neoplastic sites of the same patient, including the primary and metastatic tumor (Figs. 1 and 3). Masses of the same granules, noted by the other authors, predominated in all cells (apart from fibroblasts) grown from the neoplastic specimens but were absent in cells grown from adult or embryonic normal specimens. In a few rare normal cells certain thread-like osmiophilic granules were observed (see [*c*], Fig. 1), but these had a more constant and smaller (70 mμ) diameter than those in the neoplastic cells. The granules in neoplastic cells (see [*c*], Fig. 3) are denser than mitochondria and, even in unfixed preparations, denser than the surrounding cytoplasm (212). They have not been observed to follow any definite pattern of localization in the cell, although they frequently appear farther out in the cell periphery where mitochondria are rarely found. Some authors (246) emphasize that, on the basis of histograms constructed from measurements of all cytoplasmic particles in human epithelial normal and neoplastic cells, the granules are independent of mitochondria in size as well as in morphology. Although the French workers consider that a complete variety of particle sizes between mitochondria and the smallest granules may be observed, they believe that, because of their smaller diameter and filamentous form, they are probably not a simple build-up or degradation product of the mitochondria. They note suggestive morphological evidence that the "ultra-chondriome" is capable of division and suggest that, although the granules bear no morphological similarity to viruses, they may be conditioned by the presence of a virus. Porter also suggests that the granules are capable of division and that their presence is somehow related to the growth process, i.e., rapidly proliferating tumor and embryonic cells possess them while in "resting" adult cells they are absent. He therefore suggests that the granules may be the site of cytoplasmic ribonucleic acid, although the presence of granules in normal white cells (191) and Selby and Berger's failure (246) to find recognizable deposits of granules in embryonic cells make this less likely. The granules may not have been observed in embryonic cells because the actual cells observed in culture were not growing sufficiently rapidly, or because too small a percentage of the cell volume was visible to permit accurate judgment, but subsequent studies (244) of thin sections of mouse and human embryonic liver have failed to reveal significant numbers of the same granules which are readily observed in hepatoma. The possibility still remains, therefore, that the granules reflect some property of neoplastic cells other than a rapid growth rate, and that the 70 mμ granules (246) may be yet another cytoplasmic component or cellular contaminant.

Extensive electron-microscopic (29–31) studies upon smear preparations of blood cells have been reported by several French authors. All cells in air-dried or alcohol-fixed preparations show a granular-fibrillar structure with the individual granules possessing diameters between 50 and 100 mμ. Rodlike and elliptical granules were seen in neutrophilic and eosinophilic leukocytes and spherical mitochondria in lymphocytes. Observation of human red blood cells (31) during osmotic and mechanical hemolysis demonstrated that osmotic hemolysis is a progressive phenomenon, while hemolysis by mechanical means and by antisera is "explosive." Agglutinated cells appear to be joined by a fine thread, presumably originating from the membrane.

The improved methods of Bernhard, Braunsteiner, and co-workers for spreading and fixing cells was applied primarily to the study of normal

and pathological white blood cells (23–25, 40, 41, 118, 190–192). Prepared at 37° C., leukocytes are well spread and show extensive pseudopods (23, 25). After osmic acid fixation the cells show the same cytoplasmic detail as cells in tissue culture. Three regions of the cell are described—a central opaque area corresponding to the nucleus, a perinuclear zone containing many osmiophilic granules corresponding to mitochondria and leukocytic granulations, and a clear peripheral zone containing only lightly staining components. Reticulocytes and the relation of thrombocytes to blood coagulation (41, 42) were also studied by this method. Marchant (162) described phase and electron microscope studies of white blood cells prepared by various types of smear technic. The action of about 30 common fixatives upon the ultrastructure of leukocytes and thrombocytes has been observed (118). All fixatives revealed a fibrillary cytoplasmic network of submicroscopic dimensions. One group of fixatives (e.g., chromic acid, picric acid, and alcohol) exaggerates this by increased precipitation or coagulation, while the other group (e.g., osmium or formol) appears to reduce the structure by showing it in only the finest dimensions.

These leukocytes were then compared to cells from blood of eight acute and three subacute cases of human leukemia (cells from infant and lymphoid leukemia did not spread sufficiently well to be studied). It was observed that the leukemic white cell does not show as many pseudopods as a normal leukocyte but does contain identical cytoplasmic particles, except that a variety of granular or granulofilamentous osmiophilic particles with diameters between 60 and 170 mμ grouped in chains or colonies appear predominantly in the leukemic cells. These granules are the same component observed in other smeared and cultured cells and discussed above.

There have been many studies of erythrocytes and erythrocyte ghosts, but the great number of artifacts inherent in these is eloquently indicated by a recent study by Latta (158). He applied a method originally described by Hall (120) to replicate the cells whole and fresh without introducing any fixation. No fine structure was revealed in the surface of the intact mammalian erythrocyte of an order of magnitude larger than 60 A. Cells osmotically hemolyzed at pH 7.0 have a thickness of about 140A or less, while sections of intact cells have a thicker outer layer varying up to 1000 A. Hemolysis in neutralized distilled water produced changes ranging from small irregularities to large round holes, while hemolysis in hypotonic saline produced no visible defects. Hemolysin and complement caused fine cracks in the surface in the course of immunologic hemolysis. Latta found that the more carefully and rapidly an erythrocyte is prepared, the smoother the surface is, and discussed the relationship between his observations and other studies of membrane structure.

Wolpers (266) described normal and pathological reticulocytes prepared by the critical-point method to retain three-dimensional form. Rebuck *et al.* (220) studied the morphology of erythrocytes of sickle-cell anemia by electron microscopy, while Yasuzumi *et al.* reported granular detail in erythrocyte nuclei (273).

The relatively structureless surface of the erythrocyte has proved to be a useful substrate upon which to visualize viruses. Dawson and Elford (74) and Heinmets (127) first took advantage of the affinity of the influenza virus for the ghosts of chicken and human erythrocytes and photographed this virus adsorbed upon ghosts. Employing this method, they were able to study the relative sizes and forms of influenza and related viruses and observe some quantitative relationships of their attachment to cell membranes. Despite the many virus-like artifacts which may appear on such structures (5), the method affords a simple way to recognize the virus, especially when purification may be a difficult procedure (91). Rhian *et al.* (223) were able to observe fine changes in the surfaces of the cells following adsorption by preparing pre-shadowed replicas of the blood smears before and after adsorption and elution of the virus.

Electron-microscopic observation of cultured cells has also been useful in the elucidation of such problems as the genesis of collagen fibers (216), the structure of neurofibrils (82) and, in particular, as will be reviewed below, for the study of the relation of virus growth to the host cell (16, 99, 186, 264). Because of the inadequacy of the tissue culture method for further elucidating some of these problems, it is indeed fortunate that the advent of improved methods for thin-sectioning has made it possible to study these same cells in whole tissue.

Thin sections of tissue.—The many cytological applications of the new thin-sectioning technics which have already been made are indicated in Table 1. Most investigators of method, fixation, etc., used liver, pancreas, kidney, or intestinal epithelium as their test object, so that there are more micrographs of these tissues available than of any other.

The particulate nature of the ground substance of liver and a filamentous component of the cytoplasm were first described by Claude and Fullam

(62), and later revealed with increasingly finer resolution by Dalton *et al.* (72), Bernhard *et al.* (27), Hillier and Gettner (134), Palade (197), and Sjostrand (250). The fibrillar component has now been observed in a variety of cells—liver (27, 72, 134, 197), kidney (252), pancreas (69, 197, 250), hepatoma (72), intestinal epithelium (69), and Ehrlich ascites tumor (245)—and is illustrated in Figures 5, 6, and 7. Dalton *et al.* noted that the quantity of this component was smaller in liver from a fasting animal and was very prevalent in hepatoma. Bernhard and co-workers (27) observed an increase in the quantity of this component in regenerating liver and in liver after feeding following a fast; they concluded that there is an exact correspondence between the quantity of this cytoplasmic structure and the basophilic substance or "ergastoplasm" familiar to cytologists. Since the location of its diminution or increase in quantity in liver appears to have special relation to the mitochondria, they suggest that the results mean that the mitochondria play a large part in its elaboration and disappearance. The fibrillar aspect of this substance in thin section led Palade (197) to consider it to consist of canalicular structures running through the cytoplasm. Dalton (69),

TABLE 1

STUDIES OF THIN-SECTIONED TISSUE

Cell type	Authors
Liver cells of human	Morgan and Mowry (1951)
Liver cells of rat	Pease and Baker (1948); Bernhard, Haguenau, and Oberling (1952)
Liver cells of guinea pig	Claude and Fullam (1946)
Liver, intestinal, renal, and pancreas cells of rat	Palade (1952)
Liver, intestinal, and renal cells of mouse	Dalton, Kahler, Striebich, and Lloyd (1950)
Liver and intestinal cells of frog and mouse	Newman, Borysko, and Swerdlow (1950)
Rat and human kidney	Gautier and Bernhard (1950)
Rat kidney	Pease and Baker (1950)
Mouse kidney	Sjostrand (1953)
Pancreatic cells of mouse	Sjostrand (1953)
Intestinal and pancreatic epithelial cells of mouse	Dalton (1951)
Epithelial cell types of mouse	Dalton, Kahler, and Lloyd (1951)
Epithelial, nervous, mesenchymal, and muscular rat tissues	Palade (1952)
Human skin	Adolph, Baker, and Leiby (1951); Pease (1951)
Rat seminal epithelium	Watson (1952, 1953); Palade (1952)
Chicken chorioallantoic epithelium	Eddy and Wyckoff (1950); Borysko and Bang (1951); Bang (1952)
Intestinal epithelial cell of Ascaris	Bretschneider (1949); Granger and Baker (1949)
Rat lung	Low (1952)
Rat cornea	Jakus (1953)
Guinea pig retina	Sjostrand (1953)
Bone	Bretschneider (1949); Robinson (1952)
Teeth	Pease (1951)
Striated muscle of rat	Pease and Baker (1949); Morgan (1950)
Striated muscle of frog	Danon and Kellenberger (1950)
Smooth muscle of Mytilus, Aeschna, and Triton	Bretschneider (1949)
Smooth muscle of Venus	Cannan (Selby) (1951)
Nerve cells	Hartmann (1952)
Nerve cells and axon of cat	Bretschneider (1951)
Nerve of rat	Fernandez-Moran (1950); Latta and Hartmann (1950); Rosza *et al.*, (1950)
Nerve of mouse	Sjostrand (1953)
Nerve of frog, human, and Loligo	Schmitt and Geren (1950)
Human erythrocyte	Bernhard (1952); Latta (1952)
Mouse erythrocyte	Newman, Borysko, and Swerdlow (1950)
Human and mouse neoplastic tissues	Gessler, Grey, Schuster, Kelsch, and Richter (1949)
Mouse hepatoma	Dalton *et al.* (1950); Selby (1953)
Rat sarcoma	Porter and Kallman (1952)
Breast carcinoma of mouse	Bretschneider (1950)
Ehrlich mouse ascites tumor	Selby (1953); Grey and Biesele (1953)
Whitefish blastula	Beams, Evans, Baker, and van Breemen (1950)
Oocytes: Ascaris, Cyanea, Coregonus	Bretschneider (1949, 1950); Beams (1950)
Arbacia egg	McCulloch (1950, 1951)
Salivary gland of Diptera	Borysko (1953); Pease and Baker (1949)
Cuticles of insects: Periplanata, Culex	Richards, Anderson, and Hance (1942)
Ciliata: Paramecium, Opalina, Isotricha	Bretschneider (1949, 1950)
Paramecium	Hamilton and Gettner (1952)
Nematocysts of Corynactis	Bretschneider (1949, 1950)
Amoeba proteus	Harris and James (1952)
Bacteria	Fernandez-Moran (1950)
Onion: root tip, cells of meristem	Newman, Borysko, and Swerdlow (1950); Bretschneider (1950); Rosza and Wyckoff (1951)
Euglena	Wolken and Palade (1952)

Bernhard *et al.* (27), Sjostrand (250), and Selby (245) suggested that since cross-sections of canaliculi are not seen the fibrillar images represent sections of ribbons or lamellae. Dalton (69) noted that the lamellar-form after fixation need not be its form in the living cell. Although originally appearing to be single fibers, later work (27, 197, 245, 250) has shown that the canaliculi or lamellae are actually double-walled. Sjostrand's measurements on the rat kidney indicate that each wall is 80 A thick and separated from each other by a 110 A space (250), as illustrated diagrammatically in Figure 5. Fibers revealed in the ground cytoplasm of the Arbacia egg appear similar to these except for being considerably thicker (1000 A wide) and not resolved as double-walled (169). This discrepancy may be due to different fixation and poorer resolution due to removal of the embedding and shadowing.

After fixation in a variety of fluids, Bretschneider found the nucleus and cytoplasm of all cells to consist of a three-dimensional reticulum of 80 to 250 A "leptones" (47, 48). Comparing this structure to that obtained from various colloidal systems similarly treated, he found that the reticular structure of the cell plasm is very regular, and often hexagonal, whereas it is quite irregular and variable in the colloids. He discussed the relation of this universally found reticulum to its oriented form in the axoplasm of nerves (47) (see below) and in the spindle region of dividing cells (46, 48) (see below). Wyckoff (269) also emphasized the gel-like structure of the nucleus and cytoplasm. The reticular nature of this structure was probably enhanced by the use of acid fixatives and by removal of the embedding, since in sections fixed in buffered osmic acid only a fine granular component of dimensions about 200 A is observed. This fine background of the cytoplasm varies greatly with fixation, however, and has not yet been adequately described (see Figs. 5, 6, and 7).

The fact that mitochondria possess a limiting membrane and some internal structure was revealed by various authors in thin sections of kidney (207), paramecium (122), seminal epithelium (256), and pancreas (195). The internal structure was variously reported as consisting of particles (207), rodlets (195), cylinders (122), and membranes (256). Palade has now reported observations upon the mitochondria in a wide variety of rat cells (198). He reported that the limiting membrane is 70–80 A thick and that a system of internal ridges protrude from the inside surface of the membrane in parallel series toward the interior. They are discontinuous in the center and leave a clear channel of structureless matrix down the center of the mitochondria. Occasional granules are seen in the structureless matrix. The same structure was found in mitochondria of all cells examined (Fig. 7). In a study of kidney mitochondria at higher resolutions, Sjostrand (250) described the limiting membrane as consisting of two osmiophilic walls 45 A thick containing a 70 A space within (see Fig. 5). He also found the internal membranes to be double-walled, with the same dimensions, and extending in parallel array completely and continuously across the interior and not necessarily in contact with the limiting membrane. He also described densely osmiophilic granules dispersed between the internal membranes. From the dimensions of the membranes he suggested that the two membranes are mainly protein, and separated by a double layer of lipid, from which interesting speculation regarding spatial arrangement of enzymes may be made. It will remain for further work to determine whether Palade's failure to observe continuous membranes across the cavity is due to fragility of the structure during fixation and embedding, or whether Sjostrand's continuous membranes result from chance cuts avoiding the central cavity of the mitochondrion. Statistical assay of results should rapidly solve the last questions. Thus it is impressive that the morphology of mitochondria has now been defined on all levels of structure from the microscopic to the molecular.

Dalton (68) reported a study of the Golgi substance in the liver and intestinal epithelium and concluded that it consists of an osmiophilic and an osmiophobic component in the principal cells of the intestine, but only of an osmiophilic component in hepatic cells. The fact that the osmiophilic component reduces osmic acid only after treatment with a combination of strong oxidizing agents is said to explain why it has not been observed in other studies. An attempt was made to demonstrate glycogen in the human liver, but the treatment necessary to reveal this unfortunately rendered the rest of the cell unrecognizable (177).

Although variously described (67, 102, 207), the brush border of the kidney has been shown at high resolution to have a honey-comb appearance in cross-section. Curved rods apparently extend from it into the cytoplasm (252). The most recent (197) study of the epithelial cells of the intestine shows that the filaments extending from the free cell surface do not extend into the cytoplasm but end on a clearly defined membrane. In one study, fine filamentous projections were observed extending from the free cell surfaces of parietal, surface epithelial, and chief cells of the epididymis and the choroid plexus (71). Although this result suggests

that all these cell borders must be concerned with secretory as well as with absorptive activities, it must be remembered that submicroscopic pseudopods may be observed on the free surfaces of cells in ascitic fluid (245), in chorioallantoic membrane (13), and in tissue culture (186), and, to a greater degree, in olfactory epithelium (36) that might, with insufficient resolution, be confused with a true filamentous border.

In sections of rat lung a pulmonary epithelium has been clearly demonstrated with the cell bodies located chiefly on the thicker alveolar walls and attenuating to form a complete covering for the walls (160).

Two investigators (1, 201) of thin sections of skin found that the prickle-cell cytoplasm appears more granular than fibrillar, while the fibrils that are seen are too fine to be identical with tonofibrils in the conventional sense. Certain stubby fibrils appear between cells but penetrate only short distances into the cytoplasm, while a delicate cell membrane is always found between cells. Jakus and Sjostrand report studies of rat cornea (144) and retina (249).

Studies have also been made with reference to specific cellular structures rather than to tissue type. Hartmann (125) found that the nuclear membrane may show variations in structure not referable to cell type; it frequently appeared double, but thinner and less complex than that in Proteus and the oocytes described above. "Coiled" nucleoli are reported by Borysko and Bang (38) and Bernhard *et al.* (28), which may be seen in every cell type and in every stage of functional activity. The nucleoli appear to contain closely wound filaments or ribbons 90–180 mμ thick.

Chromosomes and mitotic figures have been difficult to study, because of the inapplicability for electron-microscopic work of conventional nuclear stains and fixatives. In sections of crayfish testis, Beams *et al.* (18, 19) showed that the whole fibrillar structure of the cell, as produced by Bouin's fixation, becomes oriented in the spindle region into the typical spindle shape with chromosomes attached to the fibrils. Studies by Rosza and Wyckoff (232, 233) and by Bretschneider (46) of neutral formalin- and osmic acid-fixed cells of the onion root tip failed to reveal any spindle structures apart from a slight orientation of the protoplasm in the spindle region. Bretschneider pointed out that the densities observed in formalin-fixed material are not the result of staining and so reflect electron densities in the original material. Both authors noted doubling of the chromosomes in prophase, while Rosza and Wyckoff reported a filamentous structure in prophase and telophase chromosomes but mentioned the possible artifacts this may be. Metaphase and anaphase chromosomes appear completely structureless. In mitotic cells of the Ehrlich mouse acites tumor, fixed in buffered (pH 7.2) osmic acid, chromosomal fibers were observed as osmiophilic fibers (Figs. 8, 10, 11), while other parts of the spindle consisted only of orientation of cytoplasmic particulates (Fig. 12). A gross periodic contouring of 0.37–0.40 μ in mouse chromosomes was observed (245) by Selby. Mitochondria were found in intimate association with chromosomes in early prophase (Fig. 9) (245).

Although tumor tissue is of obvious interest to students of ultra-structure, most of those interested in sections of this tissue have found it necessary first to understand the ultrastructure of normal tissue. Earlier studies of sections of Rous sarcoma (108), mouse mammary carcinoma (108), human carcinoma (109), and heart muscle from a tumor-bearing mouse (149) emphasized the denser cytoplasmic particles observed and implied that these are of viral character and involved in the malignancy of the cell. As Porter and Kallman (212) pointed out, the majority of micrographs show clearly that the fine structure of the cell has suffered from fixation and other procedures used to prepare them, and little attention has been paid to normal dense cytoplasmic components of the same size range or to comparable photographs of normal cells. Porter and Kallman review the difficulties inherent in interpretation of sections of tumor cells and show a photograph of a section of Jensen rat sarcoma. In this cell, the same cytoplasmic components are visible as in a comparable cell in tissue culture, including the osmiophilic particles called "growth granules." These have also been seen in sections of mouse hepatoma (244). Observations upon cells of the Ehrlich mouse ascites tumor are incomplete but suggest that virus-like deposits may be found in place of growth granules.[2] It is significant that both granular and virus-like structures present in cultured tumor cells have now been observed in sections of tumor tissue, but there is yet much study to be made of cells in other pathological conditions (inflammation, regeneration, degeneration, infection, etc.) in order to establish the true relation of the granular particulates to neoplasia.

With the visualization of individual virus particles in suspensions came the immediate possibility of seeing them within the cells they infect. Appropriately enough, since tobacco mosaic virus was the first virus to be observed in purified form, it was the first one to be seen within an infected

[2] C. Cannon Selby, unpublished data.

cell. Black *et al.* (34) photographed sections cut through infected tobacco leaves and visualized fibrous masses within infected cells and possibly within chloroplasts. They identified the individual filaments in these masses with the virus particles and noted that their great length (greater than 2800 A) suggests an end to end arrangement of the virus rods *in situ*.

Bang (13) has summarized the available information on the reaction of epithelial cells of the chicken chorioallantoic membrane to influenza and Newcastle disease viruses. Filaments and spheres of virus, distinguishable from normal cellular "microvilli," projecting from the free cell surface into the allantoic cavity are the only visible changes in the cell until late in infection. These epithelial cells do not show mitochondrial lesions at this stage, but white cells migrating into the area show large ballooned mitochondria in which particles, possibly resembling the virus, are seen. Eddy and Wyckoff (88) reported that vacuolization changes within the cells may represent the later stages of cell necrosis. Murphy *et al.* (186) studied three strains of influenza in tissue culture as well as in sections of chorioallantoic membrane and described normal surface projections and extruded products of degenerating cells in culture which are similar to those observed in sections of other free cell surfaces (71) and which may easily be confused with influenza filaments.

In chorioallantoic membrane infected with fowl pox virus (15, 269) clumps of particles, similar to those seen in purified suspensions, were easily recognized at the surface and within the diseased epithelium, but not necessarily within any recognizable inclusion body. Vacuolization and granulation of the cytoplasm was noted but was difficult to associate with virus. Bang and Gey (15) concluded that the virus may exist freely in the cell's cytoplasm before the inclusion is formed, that many vacuoles occurring in the cytoplasm of the cell are distended mitochondria with virus nearby, and that the nucleolus of an infected cell may show an even more regular ribbon-like structure than in a normal cell.

Similar mitochondrial changes were found with vaccinia and herpes simplex infection (13). The latter virus was never recognized within the cell, while the brick-shaped vaccinia was shown free in the cytoplasm and in a recognizable inclusion body when bound together in masses. Melnick *et al.* noted that virus particles in early stages of infection are adjacent to the nuclear membrane and suggested that the nucleus plays a role in their formation (171). Vaccinia virus also was recognized in tissue culture (264).

Of the neurotropic viruses, the only reported study of host-cell relationship is that of equine encephalomyelitis (16). Infected tissue cultures yield large amounts of virus, easily identifiable in the electron microscope, which appear to replace the cell's ground substance before the cell completely dissociates. The same phenomenon has been observed in infection of tissue cultures of mouse sarcoma 180 with West Nile encephalitis virus (Fig. 4).[2]

Two virus-induced benign tumors of man, molluscum contagiosum and warts, have been studied in thin sections. Blank (35) showed, in formalin-fixed sections, that areas known, from light microscopic studies, to reflect successive stages of virus multiplication and to contain the cytoplasmic DNA are actually masses of brick-shaped virus particles. Melnick *et al.* (171) further studied the structure of the inclusions and noted that the trabeculae appear segmented and appear to cut out into rounded structure, larger than the elementary virus particle. They suggested, on the basis of these photographs, that the mature virus is formed from the cytoplasmic matrix of the cell (which eventually becomes reduced to trabeculae) by a process of segmentation into provirus followed by their condensation into mature (smaller) virus. The observations on internal structure of the mature virus were not supported by Blank. Similar formations of provirus have now been shown in inclusions of vaccinia-infected cells (104), while in the case of an insect virus (33) multiplication and development of the 20-mμ virus particle take place within 160-mμ polyhedra contained in the nucleus.

Muscle.—Muscle has long been a favorite object of study for morphologists, because of the close relationship between its morphology and functional activity. Early studies of this tissue with the electron microscope served primarily to assay various preparative technics rather than to reveal new structural detail (224, 247). The great advances in morphological and concurrent physiological and x-ray diffraction studies made between this time and 1949 (9, 145, 238) and 1952 (258) are the subject of comprehensive reviews to which the reader is referred for detailed discussions. Observations upon the purified muscle proteins are included in these reviews and will not be discussed here.

The observations of Hall, Jakus, and Schmitt (121, 239) upon formalin-fixed fragments of skeletal muscle were the first to demonstrate that individual myofilaments, about 50 to 250 A in width, extend continuously in relatively straight lines through the isotropic and anisotropic bands in the contracted and extended state. These studies and

others (84, 85, 231) further illustrated that the A substance and the M and Z bands are structureless material extraneous to the filaments. A region of lower density in the A substance, called the H disk, has also been shown (121, 224). It is generally concluded that the Z and M bands perform a structural function in holding the myofibrils together. Various authors suggest that potassium (121), chloride, and ATP (85) are localized in the bands, while the great mass of these bands, as visible in fragmented myofibrils, suggests that more than inorganic salts are concentrated in these bands of such functional importance.

A 400 A period within each myofilament was predicted from x-ray diffraction analysis and was later shown in electron micrographs (239). Draper and Hodge (85) noted that the period varies inversely with the degree of shortening of muscle and as a result of microincineration studies suggested that these fine bands are due to a periodic localization of magnesium and calcium. Rosza *et al.* (231) suggested that the fine bands reflect a material superimposed upon the filaments and reported that the filaments have a constant width of 100 A rather than a variable one as suggested by the earlier authors. They emphasized the similarity between the width and striations of isolated F-actin and that of the myofilaments after the repeated washing which removed the 400 A bands. In a study on mechanically disintegrated insect wing muscle and crab leg muscle, Farrant and Mercer (93) clearly demonstrated that the periodic nodulation of the filaments may be removed by extraction in salt solution and that the filament remaining is of constant diameter, smooth, and structureless. High resolution photographs of unextracted filaments suggest the interpretation that they consist of a core of one protein overlaid with another. Filaments from arthropod muscle have an irregular period, while those from toad and rat are regular. Following Rosza *et al.* (231) these authors suggested that the basic filament is F-actin, while the salt-soluble covering contributing to the nodular appearance is myosin.

Studies on fragmented fibrils could not reveal much significant information regarding the three-dimensional orientation of these in the muscle, or of their relationship to the sarcolemma. Jones and Barer (146) and Reed and Rudall (222) described the sarcolemma as a non-filamentous membrane characterized by numerous spots 400 A to 1000 A in diameter, while others (231) also observed thin sheets with a granular surface which they presumed to be the sarcolemma, in association with certain collagen-like threads. In the first extensive study (205) of thin-sectioned muscle, hollow cores were observed in myofibrils in transverse section of lyophilized muscle. Although these cores were shown to be strongly osmiophilic, the authors concluded that the myofibril is a myofilamentous wall surrounding an aqueous core. Further studies of transverse sections of skeletal and smooth clam muscle and rabbit psoas muscle (178), affording considerably higher resolution, indicated that in formalin-fixed, osmic acid-fixed, and unfixed muscle the myofibril is solidly packed with myofilaments with diameter identical to that of isolated myofilaments. These sections of rabbit skeletal muscle indicated a hexagonal array of filaments, in cross-section, while those of clam muscle (57) indicated a greater tendency to align in two-dimensional sheets than in true hexagonal close-packing. In osmic-acid and unfixed material there is a clear membrane-like boundary separating myofibrils (not evident after formalin fixation) which, in clam adductor and in mouse skeletal muscle, is continuous with the sarcolemma membrane surrounding the fiber. In clam muscle, this sarcolemma shows great variation in thickness, suggesting that an amorphous substance is contained within it (57).

The fine 400 A striation has not yet been shown in longitudinal sections, but further study will show whether or not the failure to observe it is due simply to lack of sufficiently high resolving power. Certain mitochondrion-like globules have been observed in muscle sections (57) and in isolates (209), which appear to be intimately associated with the filaments and may, or may not, be in close proximity to the cell nucleus.

A specialized muscle fibril, found only in molluscan muscle, has, however, been of great interest because of a direct analogy between its structure and that of the myofibril. It was first noted by Schmitt *et al.* in x-ray diffraction studies (239) and later observed in the electron microscope (121). The electron-optical image is exactly as predicted from diffraction analysis: an unusual two-dimensional array of phosphotungstic-acid-staining nodes with a fundamental spacing of 145 A which is more conveniently related to a larger fiber-axis period of 725 A.

Nerve.—Electron-microscopic studies of the axon (myelin) sheath have served mainly to confirm the results of polarized light and x-ray diffraction analysis. The latter had indicated that the sheath is composed of concentrically wrapped layers of mixed lipids alternating with thin, possibly unimolecular, layers of protein material. Fragmented lamellae, presumed to originate from the sheath, were observed in fixed and fragmented

nerves by Sjostrand (248), DeRobertis and Schmitt (80), and Fernandez-Moran (96), and by the last author in thin frozen sections of medullated nerve (95). Moran concluded that the unit lamellae are 50 A thick and have a variable granular surface structure 50–100 A high. After alcohol extraction the lamellae are only 30–40 A thick. He discusses possible relationships between the lamellae, of average thickness of 80 A seen in thin section, and those of 180 A thickness predicted from polarized light and x-ray diffraction analysis. Most recently Sjostrand has described the fine structure of the sheath as revealed in thin sections with a resolution of 20 A, confirming and extending the earlier results (251). Fernandez-Moran (97) reported that submicroscopic unmyelinated nerves, 0.1 to 1.0 μ thick, consist of a thin sheath formed by a single tubular membrane containing one (in the smallest) or several filaments 1000 A in diameter.

The axon (axoplasm) has been more difficult to study due to the difficulty of determining the precise localization in nerve of any structures revealed in fragmentation studies, and the fact that axon proteins account for only 3 per cent to 4 per cent of the net weight of the fiber (238). Considerable discussion has arisen concerning the identity of certain fibrillar structures originally called "neurotubules" (80) and presumed to be related to the neurofibrils observed in fixed and stained preparations at the limit of resolution of the optical microscope. These were first observed in vertebrate and invertebrate nerve fragmented by different methods and appeared as double-edged fibrils with diameters ranging from 200 to 600 A. Later Schmitt (238) reported that, contrary to original reports (81), the filaments could be found in degenerated nerves and suggested they were actually collagen filaments from the connective tissue sheath. Since fragmentation technics are not suitable for establishing the localization of any component, several authors have investigated this point by the use of thin-sections. Most authors (97, 230, 240) find filaments of 100–200 A diameter with nodose contour in the axon and typical collagen filaments and with double-edged appearance in the surrounding connective tissue sheath. Two authors (230, 240) studying nerve sections from which the methacrylate embedding was removed considered that axonic filaments are arranged in a reticulum. Others (97, 208) find the filaments in arrays parallel to the axis. DeRobertis *et al.* studied paraffin-celloidin embedded formalin-fixed or frozen-dried sections of rat and toad sciatic nerve and found the axonic filaments in compact parallel array without nodose contour and with the double-edged appearance associated with the original "neurotubules" (79). These authors (82) have further studied nervous tissue cultured from chick embryos and observed cytoplasmic filaments resembling neurotubules to organize from microvesicles during fibrogenesis, and these in turn to organize into typical neurofibrils.

The divergence of observations upon these axonic filaments illustrates pointedly the various artifacts of preparation which we have discussed above. Most authors unfortunately found it necessary to remove the embedding from the sections, so that there was considerable opportunity for the sectioned filaments to change their position during immersion in the various solvents and during the following drying procedure, while the sensitivity of axonic filaments to fixation and embedding treatment is evident.

Most authors are in agreement concerning the existence of a neurolemma which is closely associated but not identical with collagen fibrils, whereas an axilemma has not been found. Rosza *et al.* (230) noted that the axon is constricted, but not interrupted, at the nodes of Ranvier, so that the filaments continue straight through the nodes without interruption. Robertson (227) reported that the axoplasm is not continuous through two invertebrate synapses.

Bacteria.—Application of the electron microscope to structural problems in bacteriology began with the earliest workers (130) and has been, until the present, the largest single field of biological application. Except in the area of bacterial viruses, electron-optical studies have served primarily to confirm light microscopic observations. Early advances in submicroscopic bacterial cytology have been critically reviewed by a number of authors (130, 150, 269) and need not be covered here.

In the past few years interest has centered upon identification of a bacterial nucleus. Areas of decreased electron density have been observed in unhydrolyzed cells of *E. coli* (147) and others (51) and in photolyzed cells of *B. subtilis* (17). These correspond to nuclear sites in stained preparations and in phase micrographs and to areas of increased density in hydrolyzed cells (150). Similar "nuclear" sites are found in Mycobacteria and Azotobacter algae (89, 151). Evidence is given that these nuclear sites are vesicular (150), but attempts to identify variations of density within these areas or to isolate chromosome-like elements (163) from bacteria have so far been equivocal. No particles corresponding to nuclear equivalents were found (259) in disintegrated bacteria. Areas of increased electron density are identified as sites of enzymatic activity similar to that of mitochondria (180, 182).

Attempts have also been made to record the morphological aspects of antibiotic activity. Bringman (52) correlated light, phase, and electron microscopic studies of *E. coli* under the action of streptomycin, aureomycin, and chloramphenical. Kellenberger and Werner (148) found that such nonspecific changes as increase in turgescence, shrinkage, and final extrusion of the cytoplasm accompany the action of streptomycin on *B. subtilis*. Others (50) found that streptomycin causes the filaments of avian tubercle bacillus to broaden and has a more delayed effect than isonicotinic acid hydrazide which increases the turgescence of the rods and inhibits filament formation.

Various Treponemas have been described (6) grossly and an interesting structure of spirally wound fibrils found within them and other spirochaetes (39). Mycobacteria (161, 263), *Proteus vulgaris* (11, 124, 263) and the bacterial cell wall (235) have been the subjects of other recent morphological studies upon bacteria and unicellular organisms. Bacterial flagella are of great interest to students of protein ultrastructure (10, 78) as well as to bacterial cytologists (59, 140, 254) since they exhibit contractility and yet need not be greater than that of a large protein molecule. Attention has recently been brought to the drying artifacts inherent in conventional preparations of these, but which can be eliminated by the critical-point method of preparation (4).

Of the many studies of bacteriophage interaction with bacteria (130) only the most recent have been able, by developing special technics (32, 189), to follow the virus through the infectious cycle. The critical-point method of preparation was necessary to show that, in the adsorptive phase, phages attach to the bacterial wall by their tails only (3). During the first half of the latent phase marginal vesicular areas replace the alternate light and dark pattern (nuclear and mitochondrial regions [180]) of the normal cell (32, 181). During the second half of the latent period, the cells fill with finely dispersed Feulgen-positive material, while granules, which are presumed to be phage provirus, may be seen in these areas with the electron microscope. Recognizable forms of mature virus then appear to replace the provirus prior to cell-bursting and the liberation of free virus particles (32, 181, 189).

Other cells and tissues.—The microstructure of spermatozoa lends itself very well to electron microscopy. Studies of bull (44), fowl (115), human (136), ram (218), snail (123), fish (234), and spider beetle (83) sperm have revealed the great complexity of structure in the tail, axial fiber, neck, and head. Grigg and Hodge (115) described a conical membranous cap, eleven axial filaments, and the absence of the usual (136) helically wound cord surrounding the axial filament in fowl sperm. Bretschneider (44) studied in detail the head structure of a variety of sperm and described the genesis of the homogeneous spiral body from the granular mitochondria. Randall and Friedlander (218) described much detail in the neck and membranous head of ram sperm and established the continuity of fibrils from the middle-piece to the head. Morphological changes involved in the transformation of the eupyrene flagellum to the complex tail of the oligopyrene spermatozoa have also been described (123).

Recent studies upon plant cells include those upon plant fibers (183) and developing plant cell walls (173, 184, 267). Preston (217) reported a comprehensive morphological study of Valonia. In thin sections of Euglena each 1-μ chloroplast was seen to be limited by a membrane and to consist of regularly oriented lamellae 25 mμ thick and separated by a 30–50 mμ spacing (265). In fragmented preparations of Spirogyra and Mougeotia (255) the individual lamellae appeared 70 mμ thick.

Layer-stripping and layer-digestion methods revealed the fibrillar structure and granular surface of earthworm cuticle (221). Cuticles have been popular objects of study since the earliest workers (86, 225). Until the advent of thin-sectioning (203), replica methods were the only ones applicable to the study of enamel (243), teeth (106, 242), and bone (228). Robinson correlated the available x-ray, chemical, x-ray diffraction, and electron-microscopic evidence concerning the structure of bone (228) and revealed that anisodiametric crystals align themselves with their axis parallel to the collagen filaments which make up one-half the organic matrix of bone (229).

In the interest of composing a review primarily for cytologists, further detail concerning these subjects (plants, cuticle, bone, teeth, sperm, etc.) cannot be given here. For the mass of valuable work upon purified viruses (130, 170, 269) and fibrous proteins (236, 237), the reader is referred to the reviews indicated. Recent important publications covering collagen (20), keratin (94), insulin (92), fibrinogen (119), and studies of blood clotting (179, 211), in which earlier references may be found, are cited here.

SUMMARY

The manner in which the electron microscope affords a higher resolving power than any other method of direct structural analysis is described. Survey of the literature relating to its first decade of availability for biological research shows that

much of this time has been spent in developing the special methods required for preparing specimens sufficiently thin, sufficiently dry, and with sufficient contrast for observation in this new instrument. Methods devised for this purpose include surface replicas, metal-shadowing, freeze-drying, critical-point drying, and thin-sectioning. Specimens have also been successfully prepared by cell-fractionation, cell-smear, and tissue culture. In the short time which has elapsed since the development of these methods, much submicroscopic tissue structure with widespread chemical and physiological significance has already been revealed.

The most striking contributions are those which extend the microscopist's vision to the molecular domain by describing such simple structures as muscle fibers, collagen fibers, and bacterial flagella in macromolecular dimensions. The essential simplicity of design in biological, as in other, matter is illustrated by the common lamellar structure of the nerve myelin sheath, retinal rods, and chloroplasts. This design is already, in the case of nerve, identified with a certain chemical composition. Although the structural integrity of the mitochondrion was in doubt until only recently, it has now been shown to possess a limiting external membrane and a system of internal membranes whose dimensions suggest a direct analogy to the protein-lipid structure of other membranes. The methods are now at hand for determining the ultrastructure of other cellular components (cilia, cell and nuclear membranes, brush borders, submicroscopic cytoplasmic inclusions, chromosomes, etc.) and thus achieving further correlations of cellular form with function.

The high resolving power of the electron microscope has also been useful in explaining certain phenomena of optical microscopy. The dependence of cell morphology upon the pH and chemical composition of the fixative and its change upon removal of the embedding have been clearly shown and indicate that it is the coarsened or vacuolated cell structures after acid or basic fixation and removal of the embedding that produce the best image under the lower resolving power of optical microscopes. For example, spindle fibers appear under the light microscope only when, after acid fixation, the cytoplasm precipitates very coarsely in a certain orientation. The electron microscope shows that, after neutral fixation, only chromosomal fibers appear, while the rest of the spindle exists only as an orientation of fine submicroscopic particulates.

Since virus particles are visible only in the electron microscope, some of the most striking contributions of this instrument have been in the field of virology. Most stages of bacteriophage-bacteria interaction have now been shown morphologically, and studies with other viruses, notably vaccinia, molluscum contagiosum, and herpes are rapidly progressing in this direction. Although influenza, Newcastle disease, and the encephalitis viruses are more difficult to study because of the existence of normal cell components ("microvilli" and microsomes) with similar morphology, the structural effects of these viruses are also becoming understood.

Following the description of normal cell ultrastructure, studies of tumor cell ultrastructure have already led to positive contributions to cancer research. Distinctions in the submicroscopic cytoplasmic inclusions found in normal and neoplastic cells have been observed by several investigators in tissue cultures, tissue sections, and blood cell smears. Studies of comparable cells in other pathologic conditions are already underway and should reveal the true specificity of these tumor cell characteristics. Chemical and metabolic studies of these structural distinctions can then follow. With the develop nent of thin-sectioning technics for routine use, such submicroscopic cellular criteria as those which have already been revealed could be applied to many practical problems in tumor classification and therapy evaluation beyond the scope of optical microscopy and pathology.

One of the first applications of the electron microscope to cancer research was the visualization of cytoplasmic deposits of virus-like particulates in tumors of proved viral etiology. The fact that such particulates have not yet been observed in other tumors points out, as clearly as other methods, that these tumors possess a different growth mechanism. The way is now open for following the development of viral and nonviral cellular components with the passage and growth of the tumor and so, with primarily morphological technics, further elucidating the proliferative mechanism of these cells.

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Fig. 1.—Electron micrograph of the periphery of an epithelial-type cell in a culture from a specimen of normal human buccal mucosa. Mitochondria (*a*), endoplasmic reticulum (*b*), and narrow (70 mμ) threadlike granules (*c*) are indicated. The edge of the cell (*d*) runs parallel to the top of the photograph. Two folds in the membrane (*e*) have dried to lie flat on top of the cell. Culture fixed in osmium tetroxide fumes overnight. Magnification ×10,000.

Fig. 2.—Electron micrograph of the periphery of an extended malignant hemoblast in a specimen of acute leukemic human blood. The perinuclear area with mitochondria (*a*) and chains and masses of granules (*b*) corresponding to Oberling's "ultrachondrioma" or to Porter's "growth granules" is visible. The endoplasmic reticulum is not revealed because of the short fixation time used. Fixed in osmium tetroxide fumes. Magnification ×8,400.

Photograph courtesy of Dr. W. Bernhard of the Institut de Recherches sur le Cancer—Gustave-Roussy, Villejuif (Seine), France.

Fig. 3.—Electron micrograph of the periphery of a malignant epithelial-type cell in a culture of a cervical lymph node containing metastases of a squamous-cell carcinoma of the mouth. Specimen was obtained from the same patient as the normal specimen of Fig. 1. Mitochondria (*a*), endoplasmic reticulum (*b*), osmiophilic granules (ultrachondrioma, growth granules) (*c*), cell edge (*d*), and folds in the membrane (*e*) are indicated. Cell fixed in osmium tetroxide fumes overnight. Magnification ×8,400.

Fig. 4.—Electron micrograph of a portion of a culture of mouse Sarcoma 180 cells 48 hours after infection with West Nile encephalitis virus. The virus titer in culture had increased 10-fold since inoculation of the virus, and the cells were beginning to show necrosis. Portion of a necrotic cell is shown in the top right-hand corner. The less opaque area (*a*) is the nucleus. The cell periphery (*b*) is rounded up and partially replaced by clumps of virus particles (*c*). Culture fixed in osmium tetroxide fumes overnight. Chromium shadowed. Negative print. Magnification ×13,000.

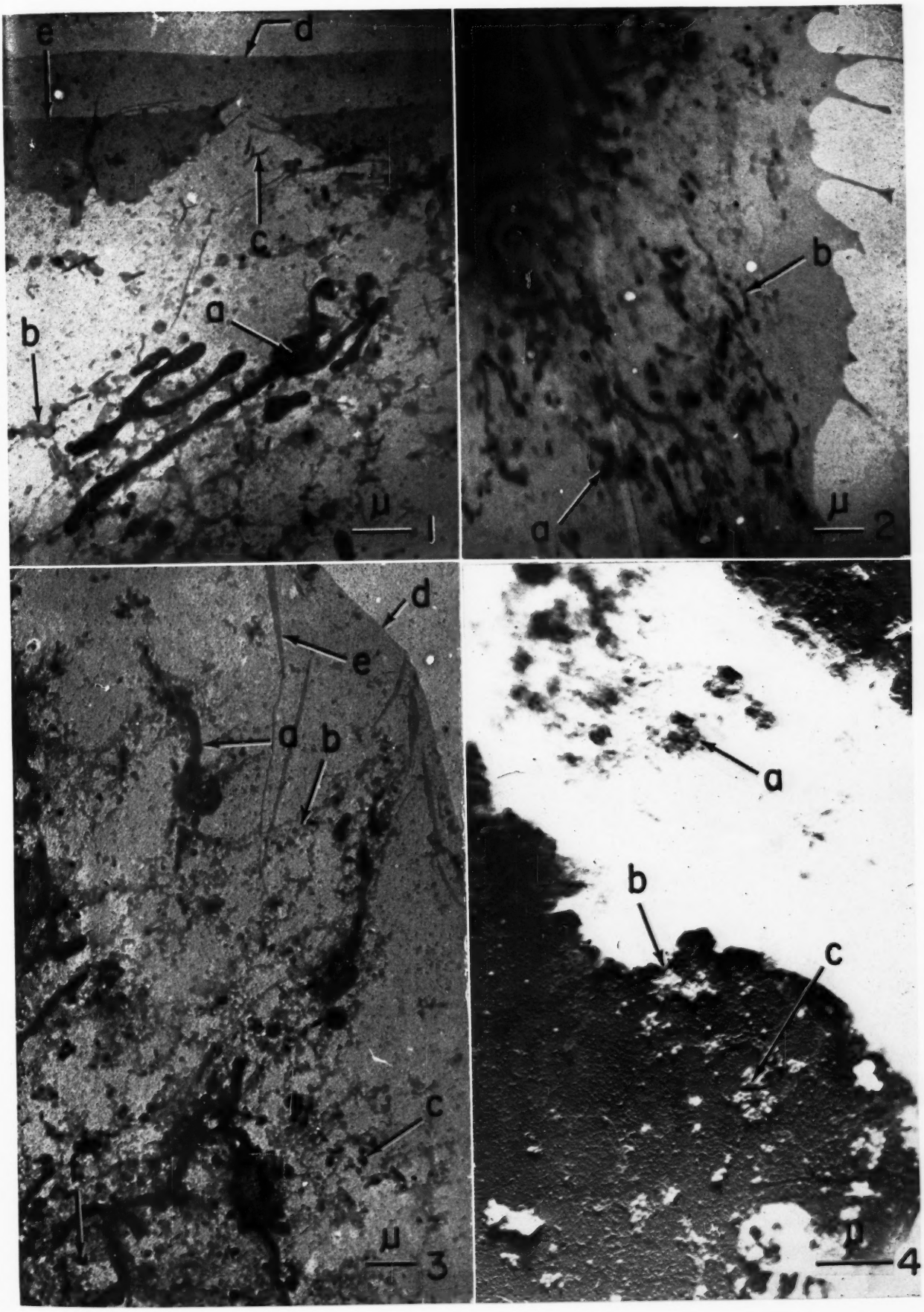
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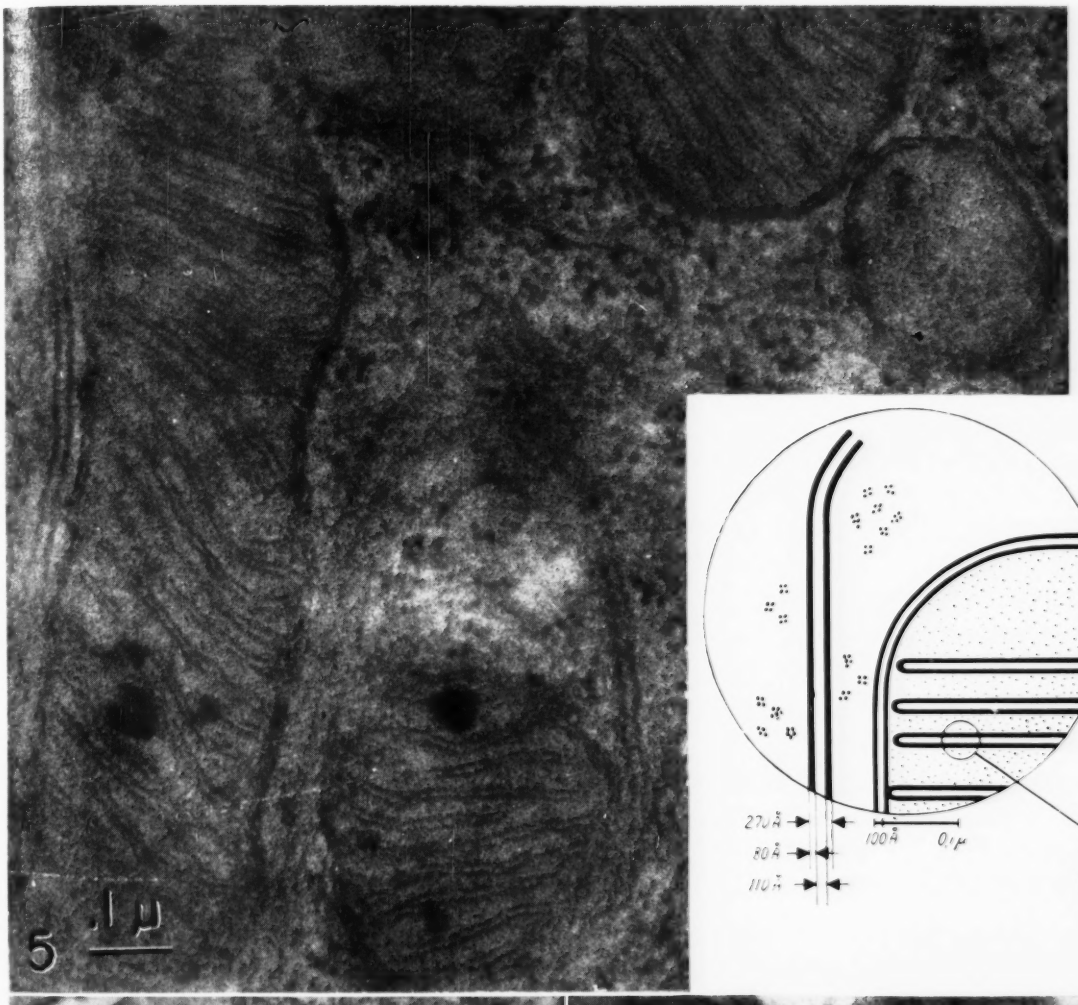
FIG. 5.—Ultrathin section through the cytoplasm of a tubular cell of the mouse kidney showing mitochondria with internal structure. Each mitochondrion shows an outer double membrane and a system of internal double membranes arranged mutually parallel. Between the mitochondria some intracellular double membranes are seen. The ground substance of the cytoplasm shows a granular appearance; the minute granules are a fairly uniform size. Magnification ×102,000. The inset illustrates diagrammatically the hypothetical organization of the mitochondrial membranes. (From F. S. Sjostrand and J. Rhodin. The Ultrastructure of the Proximal Convoluted Tubules of the Mouse Kidney as Revealed by High Resolution Electron Microscopy. *Exper. Cell Research*, in press, 1953.)

Illustration obtained through the courtesy of Dr. F. S. Sjostrand, Karolinska Institute, Stockholm, Sweden.

FIG. 6.—Ultrathin section through the cytoplasm of a hepatic cell in the liver of a full-term mouse embryo. This view at low magnification shows the cell membrane (*a*), mitochondria (*b*), and intracellular membranes or canaliculi (*c*) oriented mutually parallel in groups. The space between adjacent cells is caused by fixation. Tissues fixed in osmic acid buffered at pH 7.4 according to Palade's procedure (197) and embedded in methacrylate. Magnification ×12,000.

FIG. 7.—Ultrathin section through the cytoplasm of a parenchymous hepatic cell of an adult rat. Note the mitochondria (*a*) with "cristae mitochondriales" (198) and the endoplasmic reticulum (*b*). Tissue prepared according to Palade's procedure (197). Magnification ×38,000.

Photograph obtained through the courtesy of Dr. George E. Palade, Rockefeller Institute for Medical Research, New York City.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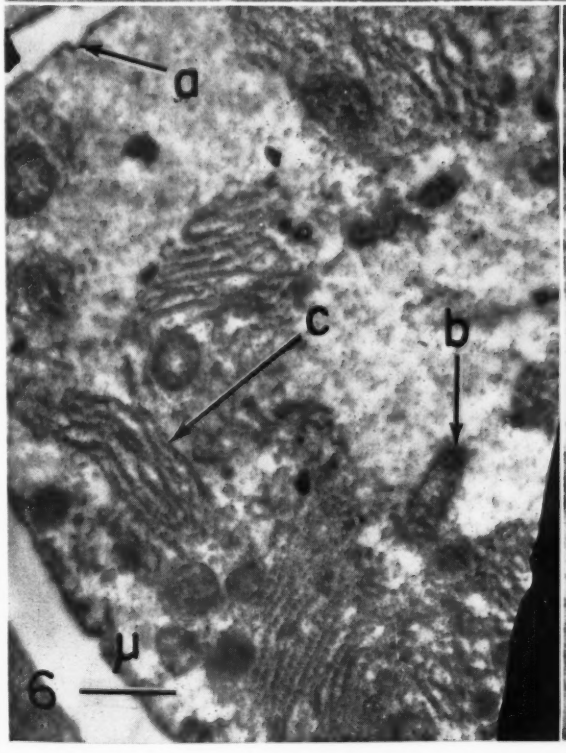
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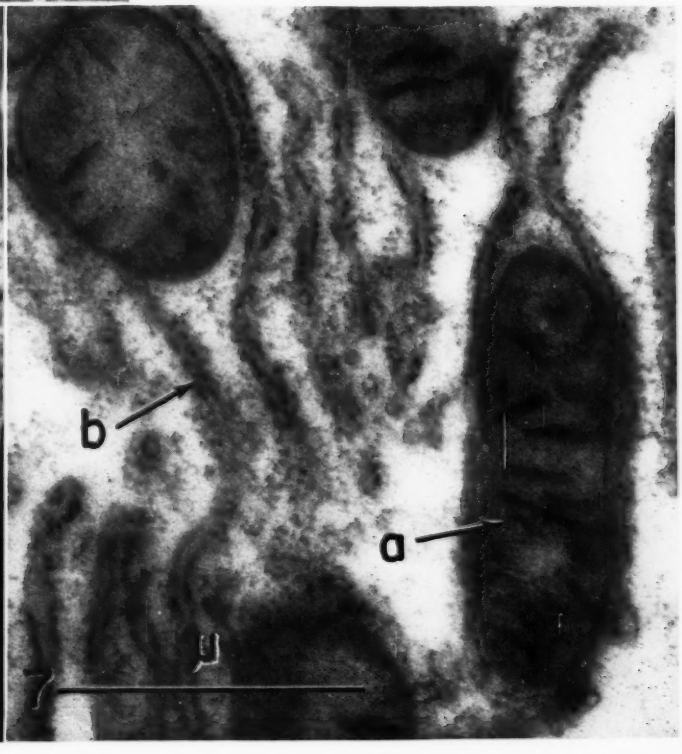
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FIG. 8.—Ultrathin section of an Ehrlich mouse ascites tumor cell removed from the peritoneal fluid of a mouse 8 days after inoculation with tumor cells. Note excellent preservation of cytoplasmic detail and mitochondria (*a*) and smaller cytoplasmic particulates (*b*) in central region. Nuclear membrane (*c*) and cell membrane with pseudopods (*d*) are indicated. Cell fixed ½ hour in 1 per cent osmic acid buffered with veronal-acetate at pH 7.0, washed in balanced salt solution, and then fixed in 5 per cent neutral formalin 12 hours. Embedded in methacrylate. Magnification ×12,000.

Photograph courtesy of Mr. Clifford E. Grey and Dr. John J. Biesele of the Sloan-Kettering Institute for Cancer Research, New York City.

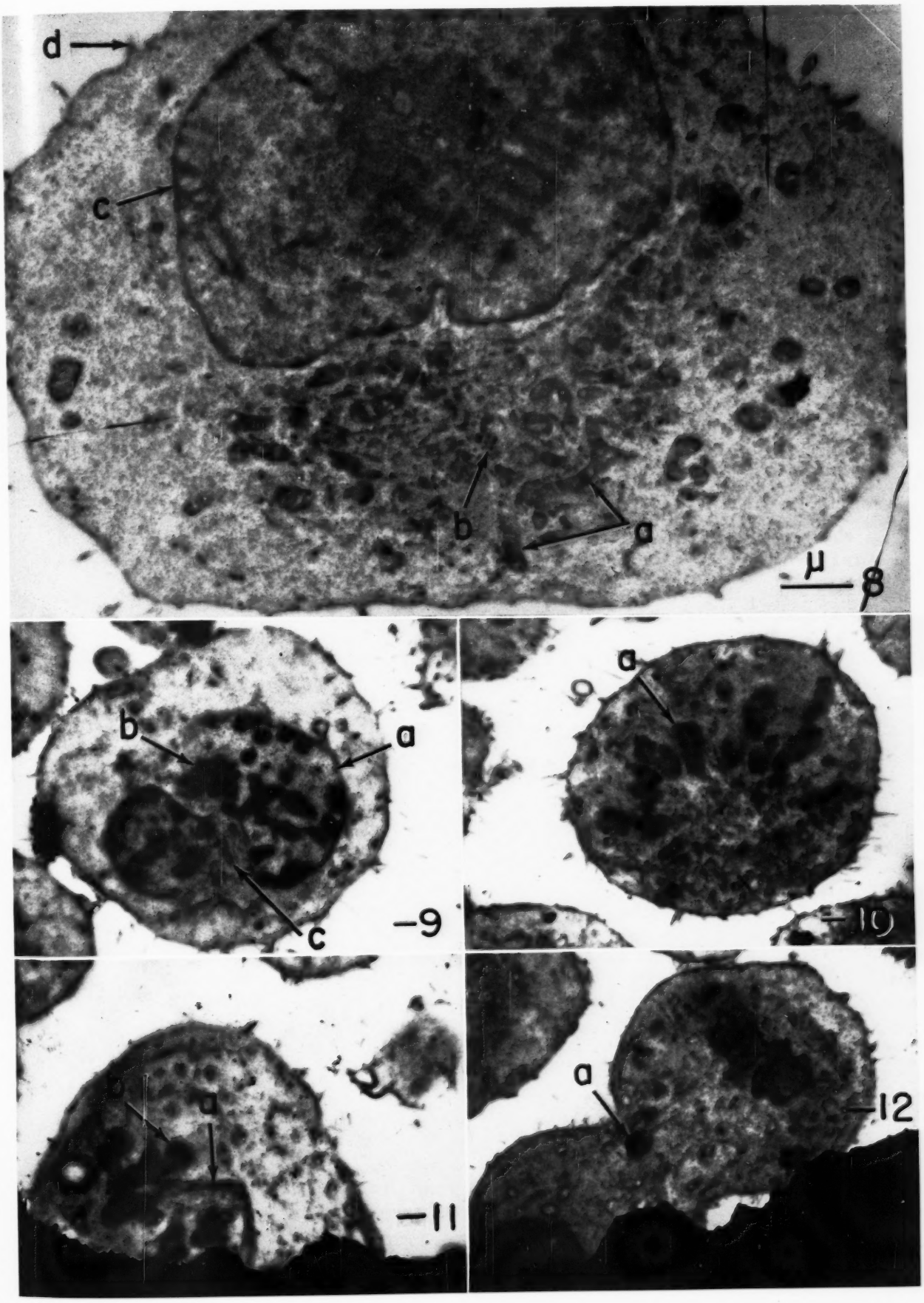
FIG. 9.—Thin section of a cell of the Ehrlich mouse ascites tumor removed from a mouse 7 days after inoculation with tumor cells. The nuclear membrane (*a*) is partially dissolved, and the chromosomes (*b*) have taken form and are in close proximity to the mitochondria (*c*). This cell, and those of Figs. 10, 11, and 12 were fixed for 45 minutes in osmic acid buffered at pH 7.2 in Locke-Ringer's solution, and subsequently embedded in methacrylate. Magnification ×4,200.

FIG. 10.—Thin section of another mitotic cell from the same specimen as that of Fig. 9. A portion of the metaphase plate is shown and the chromosomes have divided longitudinally (*a*). Magnification ×4,200.

FIG. 11.—Thin section of another mitotic cell from the same specimen as that of Fig. 9. The cell is in anaphase with the chromosomal fibers (*a*) and separating chromosome (*b*) visible. No other spindle structure is evident. Magnification ×4,200.

FIG. 12.—Thin section of a cell from the same specimen as that of Fig. 9 in telophase. The cytoplasmic bridge is being constricted and nuclei are beginning to reform. A free fat droplet is indicated (*a*). There are no indications of interzonal fibers, apart from the general orientation of the small particulate components of the cytoplasm in this region. Magnification ×4,200.

(Figs. 9, 10, 11, and 12 from C. C. Selby. Electron Micrographs of Mitotic Cells of the Ehrlich Mouse Ascites Tumor in Thin Section. *Exper. Cell Research*, in press, 1953.)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Pituitary and Adrenal Factors Involved in Azo Dye Liver Carcinogenesis

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Many investigators have noted that the pituitary exerts a direct effect on carcinogenesis and also on the growth of tumors. Korteweg and Thomas (5) found a considerably lower incidence of carcinomas in hypophysectomized mice treated with 3,4-benzpyrene than in treated controls. These investigators, however, were of the opinion that the difference between hypophysectomized mice and controls was more quantitative than qualitative. Moon and associates (8–10) have reported that prolonged administration of growth hormone in female rats resulted in tumors of the lymphatic tissues, reproductive organs, and adrenals. In contrast, no tumors were evident when similar studies were carried out in hypophysectomized rats (11). More recently, Moon, Simpson, and Evans (7) have indicated that implantation of methylcholanthrene failed to induce tumors in hypophysectomized rats. Results obtained from this laboratory suggest that the carcinogenic action of 3′-methyl-4-dimethylaminoazobenzene (3′-Me-DAB) may be effectively inhibited by hypophysectomy (3, 4).

From the above reports it is evident that hypophysectomy exerts a considerable influence on the carcinogenic process. Conflicting results have been reported as to the effect of the pituitary on the growth of established tumors or on the development of transplanted tumors. The growth of many tumors is inhibited, while other tumors continue to grow following hypophysectomy (14, 15). Loeffler (6) has reported that fibrosarcomas could be successfully transplanted to hypophysectomized rats. Gardner (1) also found that spontaneous mammary tumors continued to develop in hypophysectomized mice.

The observation from this laboratory that hypophysectomy inhibits the formation of hepatomas in rats fed diets containing 3′-Me-DAB was the stimulus for the present study. Several pituitary and adrenal hormones, as well as other hormone preparations, were injected into hypophysectomized, azo dye-fed rats in order to ascertain the particular function of the hypophysis involved in cancer induction.

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METHODS

Eight groups of hypophysectomized male albino rats[1] (200 gm.) were maintained on a synthetic basal diet containing 0.06 per cent 3′-Me-DAB (2). Initially, twelve animals were assigned to each group. The animals were kept in wire-bottomed cages in a thermostated cubicle with the temperature regulated between 24° and 26° C. Food and water were given ad libitum, and food intakes and body weights were recorded weekly.

Each group was injected subcutaneously every 48 hours with hormone preparations as follows: *Group I:* Two units ACTHAR gel for 11 weeks and 2 units H.P. ACTHAR gel for remainder of study (Armour & Co., Chicago). *Group II:* Two mg. of an equine anterior pituitary corticotrophin prepared in this laboratory. This preparation had approximately 0.3 U.S.P. units/mg as determined by the Sayers assay; however, it fully maintained the adrenals of hypophysectomized rats (12). This was suspended in gelatin. *Group III:* Two mg. of cortisone acetate—aqueous suspension (Cortone Acetate, Merck & Co., Rahway, New Jersey). *Group IV:* Control animals, 1 cc. physiological saline. *Group V:* Three mg. testosterone cyclopentylpropionate in sesame oil. *Group VI:* Two mg. of desoxycorticosterone acetate, aqueous suspension. *Group VII:* Two mg. of crude equine anterior pituitary extract containing gonadotrophin. This fraction (1 mg/rat/day) maintained testicular size in hypophysectomized rats up to 21 days; however, no further assays were carried out.

After 9 weeks several animals from each group were sacrificed by ether anesthetization. Sections of liver along with other tissues and organs were fixed in 10 per cent formalin.[2] Rats from each group were also sacrificed after 14 and 21 weeks.

A control series was included in this study. Normal intact male rats of the Sprague-Dawley strain (approximately 200 gm.) were maintained on the synthetic basal diet containing 0.06 per cent 3′-Me-DAB. Groups of rats were injected subcutaneously every 48 hours with ACTHAR gel, equine anterior pituitary corticotrophin, testosterone, DOCA, or an equine anterior pituitary extract containing gonadotrophins in the

[1] Sprague-Dawley strain. Purchased from Hormone Assay Laboratories, Inc., Chicago. There was a 2-week interval between the time of hypophysectomy and the initiation of azo dye feeding.

[2] Histological studies are being carried out by one of the authors (H. L. Richardson, Sloan-Kettering Institute for Cancer Research, New York). Results of these studies will constitute a separate report.

same dosage as the hypophysectomized series. The azo dye diet of Group III was supplemented with cortisone acetate at a level of 0.01 per cent. Group IV was fed an azo dye diet containing 0.25 per cent thiourea. Group VIII consisted of pair-fed controls. These rats were maintained in separate cages and were fed the same quantity of the dye-containing diet as consumed by the hypophysectomized dye-fed animals. Animals from these groups were sacrificed at 8 and 13 weeks. Gross observations were made, and tissues were fixed as in the hypophysectomized group.

RESULTS AND DISCUSSION

In confirmation of previous findings from this laboratory (3, 4) it was observed that hypophysectomized rats fed diets containing 3′-Me-DAB did not develop liver tumors (Group IV, Table 1). Livers from animals fed this diet for 21 weeks or 28 weeks were still normal in appearance. Somewhat lower food intakes were observed in these rats; however, diet alone was probably not responsible for this inhibition of liver carcinogenesis. A group of pair-fed, normal animals, maintained under the same conditions and restricted to the same food intake as the above hypophysectomized rats, exhibited the usual liver damage after 10 weeks, and tumors were present at 13 weeks (Group VIII, Table 2).

From Table 1 it may be observed that administration of fractions with adrenocorticotrophic properties partially restored the carcinogenic activity of 3′-Me-DAB in livers of hypophysectomized rats (Groups I, II, and VII; Table 1). Animals from Group I were injected every other day with ACTHAR gel for 11 weeks, and a different corticotrophin—H.P. ACTHAR gel—was used for the remainder of the study. Rats sacrificed after 9 weeks exhibited no gross liver changes. At 14 weeks some livers were cirrhotic, and at 21 weeks all livers were cirrhotic and some tumors were evident. Administration of an equine anterior pituitary extract with adrenal corticotrophic activity (Group II, Table 1) also resulted in cirrhosis of the livers of the hypophysectomized rats at 14 weeks and 21 weeks. A hepatoma was present in one animal of this group at the latter period. A crude equine anterior pituitary extract containing gonadotrophins resulted in liver cirrhosis at 9 weeks. At first it was believed that the pituitary gonadotrophins may be involved in azo dye carcinogenesis. Subsequent studies have revealed, however, that this fraction contained corticotrophic activity, since the adrenals of the hypophysectomized rats injected with this preparation were not atrophied. The testes of these animals were atrophied after 14 weeks. It seems more logical to correlate the liver damage with adrenal corticotrophin than with gonadotrophins.

Administration of cortisone, DOCA, or testosterone had no appreciable effect in altering the carcinogenicity of the azo dye in the hypophysectomized animals. Livers from these animals appeared normal even after 21 weeks on the azo dye regimen. Food intakes for the various hypophysectomized groups averaged from 7 to 8 gm/rat/day. Most of the groups in the hypophysectomized series lost from 10 to 35 gm/rat during the 21-week period. Administration of the pituitary extracts containing either corticotrophin or the gonadotrophins resulted in some increase in body weight (Table 1).

Groups of normal rats fed the diets containing azo dye were injected with the same hormone preparations as reported for the hypophysectomized series. It is evident (Table 2) that administration of the various preparations did not enhance or inhibit the carcinogenic activity of azo dyes to any great extent. ACTH (Group I), equine pituitary extract (Group II), DOCA (Group VI), or the gonadotrophic fraction (Group VII) had no observable effect on the course of azo dye carcinogenesis. The livers of these animals were cirrhotic after 8 weeks of dye feeding. After 12 weeks the livers were cirrhotic and enlarged, and hepatomas were present. Cortisone (Group III, Table 2) delayed the onset of liver damage in the normal rats, since the livers appeared normal after 8 weeks. Usually this period of time is sufficient to induce considerable liver damage. Because of the implication of the adrenal in the carcinogenic process, as evident from the hypophysectomized series, it was thought that cortisone would result in adrenal atrophy and possibly inhibit carcinogenesis. Symeonidis and associates (16) found that adrenalectomy inhibited somewhat the carcinogenic action of 4-dimethylaminoazobenzene. Of more interest was their observation that administration of DOCA completely inhibited the action of this azo compound. In this respect, our results are not in full agreement with those of the above investigators, since a high incidence of liver tumors was observed when diets containing 3′-Me-DAB were fed to animals treated with either cortisone or DOCA. The difference may possibly be attributed to the fact that the 3′-Me-DAB has a greater carcinogenic activity.

Thiourea was included in the diet of one group of rats to ascertain the role of the thyroid in the azo dye process. This drug retarded the carcinogenic process by several weeks, since no tumors were evident after 12 weeks on the azo dye diet. However, after 16 or 21 weeks, hepatomas were evident. Thiourea, at the level included in the diet, appeared to be toxic, which fact could account for these results. Testosterone (Group V, Table 2)

TABLE 1

THE EFFECT OF HORMONE PREPARATIONS ON THE CARCINOGENIC ACTION OF 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE IN HYPOPHYSECTOMIZED RATS*

GROUP†	AV. WT. CHANGE‡ (gm.)	AV. FOOD INTAKE (gm/rat/day)	GROSS OBSERVATIONS AT TIME OF SACRIFICE				
			No. rats	9 weeks	No. rats	14 weeks	No. rats	21 weeks
I. ACTH§	−22	7	2	Normal livers, adrenals enlarged	4	Livers from three animals were mildly cirrhotic, 1 normal	6	Livers cirrhotic and enlarged. Two animals with hepatomas.
II. Equine pituitary extract containing adrenal corticotrophin	+12	8	2	Livers granular in appearance, adrenals normal.	4	Three livers mildly cirrhotic (one rat with pituitary tag, liver cirrhotic, no hepatoma)	4	All livers cirrhotic. One animal with hepatoma
III. Cortisone	−35	7	2	Livers normal, adrenals atrophied	2	Livers normal, adrenals atrophied		
IV. Control (saline)	−13	7	2	Livers normal	4	Livers normal	2	Livers normal#
V. Testosterone	−10	8	1	Liver normal	4	Two livers showed slight granularity, two normal	2	Livers normal
VI. DOCA	−12	7	2	Livers normal	4	Livers normal	3	Two livers granular in appearance, one normal
VII. Equine pituitary fraction containing gonadotrophin	+23	8	3	Livers mildly cirrhotic. Testes and adrenals enlarged.	3	Two livers showed granularity, one normal. Testes atrophied	2	Livers cirrhotic, testes atrophied.

* Hypophysectomized male rats, Sprague-Dawley strain. Hormone Assay Laboratories, Inc., Chicago.

† All groups fed purified diet containing 0.06 per cent 3′-methyl-4-dimethylaminoazobenzene (2).

‡ Average weight change after 21 weeks on the diet containing the azo dye.

§ ACTH-ACTHAR gel, first 11 weeks, ACTHAR (HP) gel last 10 weeks, Armour & Co., Chicago.

Three animals of the control group were maintained on the diet containing the azo dye for 28 weeks. Livers of two of these rats were normal in appearance. Mild cirrhosis was evident in the liver of the third rat; however, a pituitary tag was also found in this animal.

TABLE 2

THE EFFECTS OF VARIOUS AGENTS ON THE CARCINOGENIC ACTION OF 3′-METHYL-4-DIMETHYLAMINOAZOBENZENE IN NORMAL RATS*

GROUP†	AV. WT. CHANGE‡ (gm.)	AV. FOOD INTAKE (gm/rat/day)	GROSS OBSERVATIONS AT TIME OF SACRIFICE			
			No. rats	8 weeks	No. rats	13 weeks
I. ACTH§	+42	12	2	Livers cirrhotic	6	Livers enlarged, severe cirrhosis, tumors in all livers
II. Equine pituitary extract containing adrenal corticotrophin	+50	13	2	Livers cirrhotic	5	Livers enlarged, severe cirrhosis, tumors in all livers
III. Cortisone	−31	11	2	Livers normal	3	Livers cirrhotic and tumors present
IV. Thiourea	−42	9	0	No animals sacrificed	6	Cirrhosis in all animals (animals sacrificed after 16 and 21 weeks showed extensive liver cirrhosis and multiple tumors)
V. Testosterone	+23	11	2	Livers severely cirrhotic	3	Livers severely cirrhotic and multiple tumors
VI. DOCA	+62	11	0	No animals sacrificed	8	Livers severely cirrhotic and multiple tumors
VII. Equine pituitary fraction containing gonadotrophins	+74	11	0	No animals sacrificed	10	Livers enlarged, severely cirrhotic, and tumors present
VIII. Pair-fed controls	+40	7–8	4	(10 weeks) Livers cirrhotic, no tumors evident	3	Livers cirrhotic, tumors present

* Male rats, Sprague-Dawley strain.

† All groups fed purified diet containing 0.06 per cent 3′-methyl-4-dimethylaminoazobenzene.

‡ Average weight change after 13 weeks on diet containing the azo dye.

§ ACTH-ACTHAR gel first 6 weeks, (HP) ACTHAR gel last 7 weeks, Armour & Co., Chicago, Ill.

gave some indication of enhancing azo dye activity. Rats sacrificed from this group exhibited more liver damage than rats from other groups. Rumsfeld, Miller, and Baumann (13) have reported that the feeding of diets containing azo dyes results in a higher incidence of liver tumors in male than in female rats. Further work will be necessary to determine if testosterone does accelerate liver damage or hepatomas. It was concluded that none of the hormone preparations or the thiourea altered the azo dye process in intact animals to any great extent.

All of the evidence suggests that the pituitary is involved in azo dye carcinogenesis. This conclusion is supported by the findings of Moon *et al.* (7), in which it was observed that methylcholanthrene failed to induce tumors in hypophysectomized rats. In addition, these investigators reported that growth hormone did not stimulate tumor formation in hypophysectomized rats (11). Results reported in the present paper support the view that the adrenal may also be involved in carcinogenesis (3, 4). Adrenal corticotrophic preparations partially restored the activity of the azo dyes in hypophysectomized rats. However, more time was required for cancer development than in intact rats. No tumors were observed before 21 weeks of azo dye feeding, while 10–12 weeks is sufficient in the normal animals. Other factors are undoubtedly involved in this carcinogenic sequence. Studies are now in progress to determine if growth hormone, thyrotrophin, or other pituitary hormones may also be involved. Chemical studies on the liver including bound dye determinations, riboflavin, and desoxyribonucleic acid analysis are being carried out to provide further data regarding the role of the pituitary and the adrenal in liver carcinogenesis.

SUMMARY

1. Groups of hypophysectomized male rats, maintained on diets containing 3′-methyl-4-dimethylaminoazobenzene, were injected subcutaneously with ACTHAR, equine pituitary corticotrophin, cortisone, DOCA, testosterone, or saline.

2. After 21 weeks livers from the above rats treated with saline, DOCA, testosterone or cortisone were still normal in appearance. Cirrhosis and liver tumors were evident in the liver of the rats injected with the adrenocorticotrophin preparations.

3. Intact rats, fed the above diet, were treated with the same hormone preparations. One group of these animals was restricted to the same food intake as the hypophysectomized rats. Extensive cirrhosis and hepatomas were evident in all these groups after 13 weeks.

ACKNOWLEDGMENTS

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Carboxypeptidases and Carboxypeptidase Inhibitor in Tumor-bearing Animals. A Possible Blood Test for Neoplasia*

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Carboxypeptidase is here defined as that enzyme, found in a wide variety of tissues, which hydrolyzes chloracetyl-L-tyrosine, with the production of acid groups and free tyrosine. The distribution and properties of this enzyme were recently described by us (1), and it was pointed out not only that two forms of the enzyme can be detected but that there can also be detected a naturally occurring inhibitor for one of these forms.

We wish to report here deviations from normalcy that we have found in this carboxypeptidase-carboxypeptidase inhibitor system in certain animal tumors and in the blood of tumor-bearing animals. We further wish to suggest the possibility that the blood changes may serve as a qualitative test for the presence of malignancy.

EXPERIMENTAL

Details of the enzyme assay appear in a previous paper (1). In general, the assay involves a 10-minute incubation at 37° C. and pH 5.5 of the tissue in question with 0.02 M chloracetyltyrosine in the presence, where desired, of 0.0088 M cysteine. Immediately after addition of the enzyme source, and again exactly 10 mintues later, aliquots are transferred into 10 volumes of absolute alcohol, and these are titrated with alcoholic KOH, in a manner somewhat modified from the method of Grassmann and Heyde (3).

That enzyme which requires cysteine for activity is designated carboxypeptidase *a* (CP*a*), and that enzyme which is active only in the absence of cysteine is designated carboxypeptidase *b* (CP*b*). Because of the frequent simultaneous occurrence of a CP*a* inhibitor (CP*a*I), the presence of CP*a* is often masked. In order to separate enzyme and inhibitor, high speed centrifugation in the cold is employed. After 30 minutes' centrifugation at 18,000 × *g* of normal tissues homogenized with 9 volumes of distilled water, almost all the enzyme is found in the supernatant phase, and almost all the inhibitor is found in the precipitate. Details of the distribution of the four components, CP*a*, CP*b*, CP*a*I, and CP*b*I, in a variety of normal rat tissues are presented in the previous paper (1).

Since it is relevant to what follows, we wish to recapitulate briefly that CP*a* is found in lung, spleen, liver, testis, stomach, intestine, heart, kidney, and brain, in concentrations ranging from 56 to 143 units/gm. The tissues above are arranged in order of increasing activity. Except for lung, which is essentially free of CP*b*, the same tissues show CP*b* activity, ranging from 49 to 171 units/gm. The order of activity does not necessarily parallel the CP*a* order of activity. In all cases, these enzyme activities are found in the supernatant phase after centrifuging. CP*a*I is found in blood cells in relatively high quantity (*ca.* 130 units/ml whole blood) and also in the precipitate fraction of all normal tissues listed above, except stomach. As indicated in (2), the source of blood CP*a*I is probably the leukocyte. CP*a*I activity ranges from *ca.* 50 units/gm of lung to *ca.* 120 units/gm of heart. No CP*b*I has been detected in any normal tissues tested. Skeletal muscle has also been assayed and found free of all four factors.

Table 1 summarizes the results obtained in the assay of a variety of animal tumors.[1] Although the CP*a* and CP*b* of all normal tissues was in the supernatant of centrifuged homogenates and the CP*a*I was in the precipitate, the possibility that the situation might be somewhat altered in tumor tissue led us to test all fractions for all components. All tumors were assayed at as early a stage as would permit sufficient material

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[1] For gifts of tumor-bearing animals, we wish to thank Drs. J. Sice, P. Talalay, T. Miyaji, and their associates of the Goldblatt Memorial Hospital, University of Chicago, and Dr. R. Schreck of Veterans Administration Hospital, Hines, Illinois.

for complete testing. When necrosis was encountered, the necrotic area was removed and not assayed.

In Table 1, approximately 20 units of enzyme inhibitor are considered the limit of accuracy of the method; smaller values are of little significance. It will be noted from the table that the tumors studied differ from the normal tissues studied in two ways. First, tumor tissue is essentially devoid of both enzymes and both inhibitors. Second, such enzyme activity as is erratically observed in tumor tissue is found in the precipitate fraction of centrifuged homogenates, rather than the supernatant fraction as in normal tissues.

Ascitic fluid also presents an interesting picture. In contradistinction to tumor tissue, including ascitic tumor cells, this fluid contains an appreciable amount of CP*a*I (twelve specimens averaged 116 units/ml). As has been noted, this is also found in normal tissues, especially blood, but in all cases it is found in, or attached to, the cellular portion of the tissue, i.e., the precipitate. Further, in two out of twelve mice bearing ascitic tumors, significant amounts of CP*b*I were found in the ascitic fluid (73 and 87 units/ml, respectively). These are the only cases, out of all tissues tested, where CP*b*I has been found. Because of the occasional use of malignant ascitic fluid as a growth agent in tissue culture fluid in place of embryo extract, this latter was also tested for the CP components. However, extract of 12-day-old chick embryos was devoid of both enzymes and both inhibitors.

It is of some interest that the kidney, heart, brain, spleen, liver, and intestine of tumor-bearing rats behave exactly like their counterparts from normal animals. The only tissue we have found to behave differently in the case of the tumor-bearing animal is blood.

Table 2 presents data on the CP*a*, CP*b*, CP*a*I, and CP*b*I of the blood of normal and tumor-bearing animals. It should here be noted, as before, that the limit of accuracy of the assay method is about 20 units. It will be seen that the contents of CP*a*, CP*b*, and CP*b*I are negligible in all groups of animals. However, while normal blood cells carry a considerable content of CP*a*I, the content of CP*a*I in the blood cells of tumor-bearing rats is quite low, and it is upon this phenomenon that we base our suggestion of a possible blood test for malignancy.

Table 2 contains the results of all assays performed on bloods of tumor-bearing animals, with the exception of five rats in which the results were considered difficult to classify because of unknown factors. Three of these five were rats in which tumors had been implanted (one Flexner-Jobling, one Walker, one Bagg) and in which the blood cells were devoid of inhibitor, but in which no tumor was grossly visible. No explanation is available for these apparent false positives, except the possibility that a more thorough search of the animal might have revealed small points of active tumor growth, perhaps metastases. No complete pathologic search was made at the time. The other two cases difficult to classify were those of two rats in which the Murphy lymphosarcoma had been implanted, had grown and ulcerated to the skin, and in which a small, firm residual piece of tissue remained at the site of implantation. These animals gave "negative" tests—i.e., their blood cells contained a normal quota of CP*a*I. That these apparent "false negative" tests were actually true negative tests is indicated by the histological finding[2] that the residual tissue represented a totally regressed lymphosarcoma. This finding is in line with the known tendency of this particular tumor to regress spontaneously.

TABLE 1

CARBOXYPEPTIDASE *a* AND *b* (CP*a* AND CP*b*) AND CARBOXYPEPTIDASE *a* AND *b* INHIBITOR (CP*a*I AND CP*b*I) IN RAT AND MOUSE TUMORS

	No. animals	Units/gm	Range
CP*a*:			
In supernatant	16	4± 5	0–20
In precipitate	24	9± 9	0–39
CP*b*:			
In supernatant	16	5± 5	0–19
In precipitate	24	12±15	0–72
CP*a*I:			
In supernatant	15	4± 4	0–14
In precipitate	16	6± 8	0–35
CP*b*I:			
In supernatant	15	4± 5	0–20
In precipitate	16	7± 9	0–38

Activity is expressed as the mean and average deviation from the mean. Tumors represented include the rat Walker carcinoma, the rat Bagg reticulum-cell sarcoma, the rat Murphy lymphosarcoma, the rat Flexner-Jobling carcinoma, a transplanted mouse hepatoma, the mouse Ehrlich carcinoma in ascitic form, and the mouse Gardner lymphosarcoma in ascitic form. "Supernatant" and "precipitate" of the ascitic tumors refer only to the tumor cells themselves. They were centrifuged out, homogenized with water, and then separated centrifugally into the usual two fractions. The cell-free ascitic fluid itself is separately discussed in text.

It is desirable, in any test such as this, to have an indication of the constancy and duplicability of the assay method. For this purpose, seven healthy rabbits were selected, and blood was withdrawn from each a total of 5 times, over a period of 2 weeks or more. The data of Table 3 indicate not

[2] We wish to thank Drs. R. W. Wissler and P. E. Steiner, Dept. of Pathology, University of Chicago, for their examination of this material.

only that the assay is readily duplicable, but also that the CP*a*I level of a given healthy animal is relatively constant.

To determine the speed with which the malignant process affects the blood picture and to measure the speed of reversion to normal after extirpation of the tumor, the following experiment was performed: Sixty Sprague-Dawley male rats were selected in the weight range of 200 ± 40 gm. Into each was implanted subcutaneously 0.2 ml. of a 10 per cent lightly ground, gauze-filtered homogenate of an actively growing Walker carcinoma. The animals were then sacrificed at selected times in groups of three, and their blood was assayed for CP*a*I. At 7 days, when the remaining animals bore tumors of approximately $\frac{1}{4}$–$\frac{1}{2}$ inch in diameter, all the tumors were extirpated. Again, groups of three animals were sacrificed at selected times, and their blood was assayed for CP*a*I. The remaining 30 rats, with tumors extirpated, were kept for 30 days as a check against the thoroughness of the extirpation technic; seven tumors were observed in these animals, including five which were noted at surgery as probably inoperable. The results of the experiment are shown in Chart 1, which indicates that the blood CP*a*I began to decrease within 48 hours after implantation and began to return to normal within 24 hours after extirpation of the tumor.

TABLE 2

CARBOXYPEPTIDASE *a* AND *b* (CP*a* AND CP*b*) AND CARBOXYPEPTIDASE *a* AND *b* INHIBITOR (CP*a*I AND CP*b*I) IN BLOOD OF NORMAL AND TUMOR-BEARING ANIMALS

Source of blood	CP*a*		CP*b*		CP*a*I		CP*b*I	
	Animals	Units/ml*	Animals	Units/ml*	Animals	Units/ml*	Animals	Units/ml*
Normal rabbits	45	1±1	4	0±0	45	310±66	4	10±10
Normal rats	2	8±4	2	2±2	23	184±18	2	0± 0
Tumor-bearing rats†	20	2±2	20	1±1	34	16±18	22	10±12

* Data presented are mean and average deviation from the mean. Figures are based on volume of plasma or packed cells; enzymes assayed in plasma, inhibitors in cells.

† Tumor-bearing rats contributing bloods include those listed in Table 1.

TABLE 3

DUPLICABILITY OF CARBOXYPEPTIDASE *a* INHIBITOR (CP*a*I) ASSAY IN RABBIT BLOOD

Animal no.	No. tests	Av. CP*a*I (units/ml whole blood)
1	5	446±12
2	5	400±10
3	5	394±20
4	5	292±12
5	5	370±10
6	5	292± 6
7	5	232±12

Data presented are mean and average deviation from the mean. Each sample was obtained from a separate blood withdrawal.

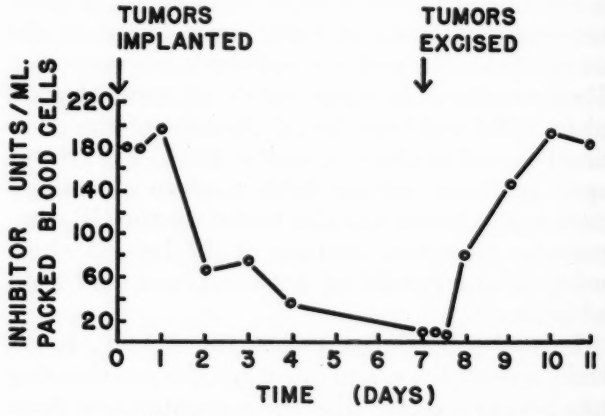

CHART 1.—Effect of implantation and excision of Walker carcinoma on rat blood cell carboxypeptidase *a* inhibitor (CP*a*I).

DISCUSSION

One of the prime deterrents to any claim of metabolic differences between tumor and normal tissue is the problem of securing the proper normal tissue with which to compare the tumor. This difficulty has been repeatedly emphasized and needs no further comment. In this work, our sole claim to "genuine" differences lies in the wide variety of normal and tumor tissues tested. Actually, all tumors tested, solid and ascitic, a variety of sarcomas and carcinomas, were totally devoid of CP*a*I, a situation which is true of only stomach and skeletal muscle, among all the normal tissues studied. Further, all normal tissues except skeletal muscle contained CP*a*, and all except lung and skeletal muscle contained CP*b;* in all cases, these enzymes were to be found in the supernatant of the homogenized tissue. In contradistinction, solid tumors had little enzyme activity, and, where present, it was in the precipitate. There seems from these data to be little reason to believe that the differences noted represent differences in embryonic origin, rather than true differences between normal and neoplastic tissue.

A possible explanation for the lack of activity of the tumor tissue is that the amounts of enzyme and inhibitor present may exactly neutralize each

other and that they may be bound in some manner that prevents their separation upon homogenization and centrifugation. This possibility was tested by shaking a sample of a tumor homogenate with chloroform, a procedure which has been found (1) to destroy CP*a*I without affecting CP*a*. The $CHCl_3$ had no effect in this case, however, so it may be concluded that the lack of enzyme and inhibitor in tumor tissue is real.

Similarly, the possibility was considered that the reason for the apparent lack of CP*a*I in the blood cells of tumor-bearing rats is that the cells also contain an equivalent amount of CP*a* firmly attached to the inhibitor. However, no enzyme activity appeared when a laked solution of blood cells from a tumor-bearing rat was shaken with $CHCl_3$. It is therefore concluded that the apparent lack of CP*a*I from such cells is real.

These data are an interesting contrast to the comment of Greenstein (4) that "there seems to be little question that tumors are, in part, characterized as much by high activity of certain proteolytic enzymes as by low activity of certain oxidases."

Our results indicate that at least one proteolytic system, far from being highly active, is decreased in activity in tumor tissue. At the same time, however, it must be pointed out that the naturally occurring inhibitor of this system is also decreased in activity. It is possible that the increased proteolytic activities in tumor tissue noted by Greenstein were simply because of loss of inhibitor. We have, in fact, some indications of such inhibitors in other proteolytic systems. The implications of such proteolytic enzyme-enzyme inhibitor systems in the cancer problem may well be profound, since such a system represents a control on protein catabolism, at least, and possibly also on synthesis.

The mechanism whereby blood cell inhibitor decreases as a tumor grows is unknown at present, but it is perhaps worthy of some conjecture. The most likely probability is that blood cells, as formed by the hematopoietic tissues, contain a normal quota of inhibitor and that tumors are able in some fashion to inactivate the inhibitor of blood cells passing through. This, of course, implies that the inhibitor decrease is much more an effect, rather than a cause of the tumor, and so diminishes the possible importance of the growth-control hypothesis suggested above. On the other hand, the fact that other proteolytic enzymes may also have naturally occurring inhibitors leads to the possibility that one may induce, another later aggravate, the neoplastic change.

Two other thoughts for future research may be recorded here. First, the possibility exists that another proteolytic inhibitor than CP*a*I may offer a more satisfactory blood test for malignancy. Second, although the preceding discussion indicates the likelihood that the inhibitor decrease may not be in any way causative, it would still appear worth while to attempt to isolate the inhibitor (or other inhibitors) and test them as therapeutic agents. If a tumor-bearing animal (or human) lacks the normal quota of inhibitor, it may be possible, by replacing the inhibitor, to arrest the malignant growth.

The clinical value of such a blood test for malignancy as is here proposed can, of course, be established only by clinical testing. Some results are presented in the succeeding paper. In addition, an attempt is being made to simplify and improve the accuracy of the carboxypeptidase assay. The present method is awkward and requires a good deal of practice before reliable results can be obtained.

SUMMARY

The enzyme-inhibitor system consisting of carboxypeptidase *a* (requiring cysteine), carboxypeptidase *b* (inhibited by cysteine), and simultaneously occurring inhibitors (CP*a*I and CP*b*I) of these two enzymes has been studied in a variety of normal and tumor tissues and in the blood of normal and tumor-bearing animals. Five different solid tumors of the mouse and rat have been found to be completely devoid of both enzymes and of both inhibitors. Two ascitic tumors of the mouse have been found free of both inhibitors but have been found to have both enzymes in the precipitate and none in the supernatant of homogenates of the ascitic cells centrifuged at high-speed. Of ten different normal tissues tested, nine had both enzymes in the supernatant, eight had CP*a*I in the precipitate, and none had CP*b*I. The ascitic fluid is the only tissue or fluid in which we have detected appreciable amounts of CP*b*I, and this was noted only erratically.

In contrast to normal rat and rabbit blood cells, we have found the blood cells of tumor-bearing animals to be essentially free of CP*a*I. This is suggested as a possible test for the presence of neoplasia.

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Clinical Evaluation of the Blood Cell Carboxypeptidase Inhibitor Test for Malignant Neoplasia

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The search for a simple biochemical, cytological, or biological test of sufficient specificity to establish the early diagnosis of malignancy has excited biologists for many years and has been the object of many investigations. To date more than 60 such "cancer tests" have been proposed. Excellent reviews of these tests have been presented by Homburger (7) and Huggins (8). With the possible exception of the serum acid phosphatase test for carcinoma of the prostate demonstrated by Gutman and Gutman (6) and the improved methods of cytological examination of fluids developed by Papanicolaou and Trant (10), none of these tests can be considered to be as reliable as a biopsy. Most recently, the tests of Huggins *et al.* (9), Black *et al.* (1), and Cliffton (2) have received considerable attention, but these tests, too, have lacked the specificity to be considered diagnostic of cancer.

We have demonstrated (5) that in the albino rat the decrease of the blood cell carboxypeptidase inhibitor (CPI) in response to an implanted tumor is extremely rapid, and that extirpation of the tumor causes an equally rapid recovery of the inhibitor concentration. This sensitive response of a blood component to the presence of a malignant lesion suggests the possibility of using this altered reaction as a blood test for malignant disease in man. A preliminary clinical evaluation of the blood cell CPI test in humans was therefore undertaken.

MATERIALS AND METHODS

Details of the assay procedure and inhibitor unitage are described in a previous paper (3). Preliminary studies demonstrated that human blood cells, presumably the leukocytes, contain an inhibitor of carboxypeptidase which is stable for at least 4 hours at room temperature and is in no way affected by the potassium oxalate used as anticoagulant throughout this study. A series of CPI assays of blood drawn from normal, healthy, laboratory volunteers was first done. Blood samples from diseased individuals were then assayed in an objective manner—i.e., the blood samples received for assay were designated merely by the patient's name, number, and location. Blood from patients from both the In-Patient and Out-Patient Departments of the University of Chicago Clinics was used. In no case was the CPI assay performed later than 4 hours after the venipuncture. The majority of the blood samples were received directly from the hospital laboratories where they had been sent for routine chemical analysis. Only in the case of the pregnancy series was a special venipuncture done for CPI assay. Thus, no attempt was made to control the conditions under which the blood was drawn.

In a previous paper (4), we have shown that the CPI titer of blood cells of common laboratory animals decreases rapidly after about 400 r of whole-body x-radiation. In this respect, then, the animal CPI response to irradiation mimics the response to malignant disease. It is, of course, impossible to measure the response of human blood cell CPI to whole-body x-radiation and difficult to correlate the CPI response to x-radiation administered locally to humans with that of whole-body irradiation administered to animals. For this reason, tests in this series were done on the blood of patients receiving all types of therapy except radiation. In those cases in which a patient had received any type of radiation or radiation-like therapy (x-ray, radium, or radon implantation, radioactive isotopes, or radiomimetic drugs such as nitrogen mustards), the results were discarded. Thus, all lowered CPI concentrations could be assumed to be due to causes other than ionizing radiation.

RESULTS

A total of 376 normal and pathological blood samples were assayed for CPI. The results are shown in Chart 1. In thirty-five patients repeat tests were done, but the data presented include only the results of the original test. In those cases where more than one disease had been diagnosed, the one of the greatest clinical significance or carrying the gravest prognosis was used as a basis of tabulation. Three hundred inhibitor units/ml packed cells was selected as the arbitrary dividing line between a positive and a negative test, because this appears to be the point at which the fewest false positive and false negative results were obtained.

In general, the blood cell CPI concentration of

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normal individuals and those with nonmalignant diseases tends to be high, while in the malignant diseases it is markedly lowered. As is true of all the other so-called "cancer tests," however, numerous results were obtained which are opposite to this general tendency. In benign tumors, diseases of the liver, endocrine diseases, granulomatous diseases, and in diabetes, there appears an especially high proportion of cases where low CPI titers were obtained. It is of interest to note that evaluation of tumors. It is therefore possible that some postoperative cases classified under malignant disease may have been clinically as well as "biochemically" cured, as measured by this test at the time it was done. If this were so, the number of false negatives would, of course, be reduced.

Further breakdowns of diagnoses in each grouping are given in Table 1, as well as the number of cases of each disease giving false positive or false negative tests. It is to be emphasized that this

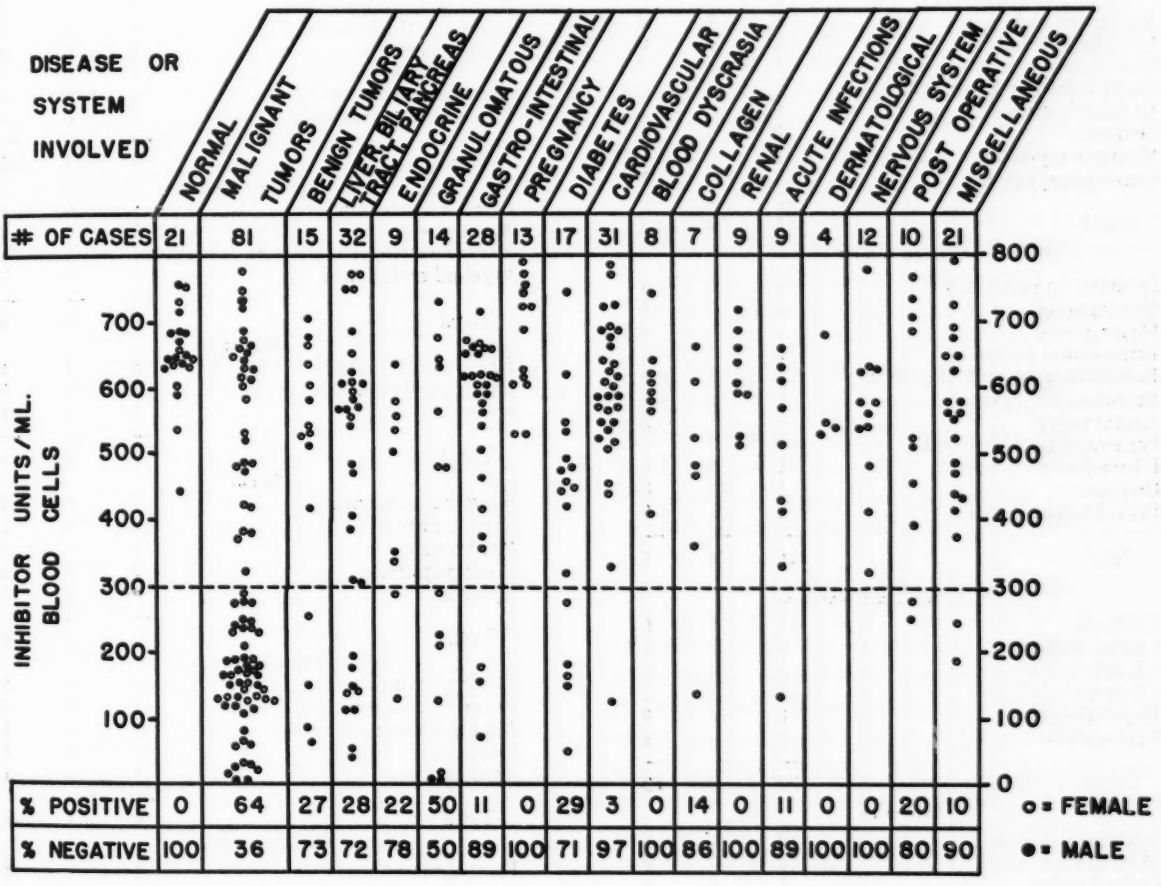

CHART 1.—Blood carboxypeptidase inhibitor titer in malignant and nonmalignant disease

many of the other "cancer tests" shows a similar high percentage of false positive results in these diseases. More undesirable, however, is the large number of patients with cancer whose blood cells gave a false negative CPI test. In all but one case (carcinoma of the pancreas), the diagnosis of malignancy was based upon histological examination of biopsy material. The limited number of patients tested and the insufficient number of repeat tests made it impossible to predict how long, if ever, after therapeutic surgery for cancer the CPI titer begins to rise, as we have observed in the rat (5). For this reason, both pre- and postoperative cases, as well as untreated cases, are tabulated under the general heading of malignant survey is of a preliminary nature, and the number of patients tested with each particular disease is, in many cases, insufficient to permit a rigorous analysis. However, it appears that only a few of the different types of malignant lesions studied are responsible for the rather high over-all percentage of false negatives. These malignancies include carcinomas of the rectum and lung, malignant melanoma, lymphosarcoma, and leukemia, as well as metastatic carcinoma of unknown primary site. The noncancerous diseases causing false positive tests are also rather limited, the chief offenders being cirrhosis of the liver, pulmonary tuberculosis, and diabetes mellitus. False positive results appear in other diseases with much less frequency.

TABLE 1

Individual Diseases Tested and Number of Positive and Negative Tests

	Total cases	Pos.	Neg.
Malignant tumors			
Metastatic carcinoma of unknown primary site	5	2	3
Carcinoma of mouth	1	0	1
" esophagus	3	3	0
" stomach	8	7	1
" colon	9	8	1
" rectum	7	3	4
" ovary	6	4	2
" cervix	2	2	0
" breast	8	6	2
" lung	4	1	3
" prostate	5	5	0
" pancreas	4	3	1
Malignant melanoma	3	1	2
Lymphosarcoma	3	1	2
Lymphoblastoma	1	1	0
Giant follicular lymphoma	2	2	0
Hodgkin's disease	1	0	1
Leukemia	7	3	4
Multiple myeloma	1	0	1
Craniopharyngioma	1	0	1
Total	81	52	29
Benign tumors			
Prostatic hypertrophy	4	0	4
Meningocoel	1	0	1
Meningioma	1	1	0
Intracranial tumor	2	0	2
Basophilic adenoma of pituitary	1	0	1
Bronchogenic cyst	1	0	1
Rectal polyp	1	1	0
Perineural fibroblastoma	1	0	1
Fibroadenoma—breast	1	0	1
Osteoma	1	1	0
Pheochromocytoma	1	1	0
Total	15	4	11
Liver, biliary tract, pancreas			
Cirrhosis	11	5	6
Cholecystitis	6	2	4
Cholelithiasis	3	0	3
Choledocholithiasis	2	0	2
Hepatitis	8	2	6
Pancreatitis	2	0	2
Total	32	9	23
Endocrine			
Addison's disease	2	1	1
Myxedema	2	0	2
Colloid goiter	1	0	1
Hypothyroidism	1	1	0
Hypoparathyroidism	1	0	1
Acromegaly	1	0	1
Hypogonadism	1	0	1
Total	9	2	7
Granulomatous			
Pulmonary tuberculosis	9	5	4
Sarcoidosis	2	1	1
Pneumoconiosis	1	1	0
Syphilis	1	0	1
Histoplasmosis	1	0	1
Total	14	7	7
Gastro-intestinal			
Ulcers—gastric and duodenal	12	1	11
Ulcerative colitis	6	1	5
Regional enteritis	1	0	1
Diverticulae	4	1	3
Appendicitis	2	0	2
Amoebic colitis	1	0	1
Functional bowel distress	1	0	1
Volvulus	1	0	1
Total	28	3	25

	Total cases	Pos.	Neg.
Pregnancy			
Pregnant	9	0	9
Post-partum	4	0	4
Total	13	0	13
Diabetes Mellitus			
Diabetes Mellitus	17	5	12
Cardiovascular			
Congestive heart failure	9	0	9
Rheumatic heart disease	8	0	8
Arteriosclerosis	3	0	3
Cor pulmonale	1	0	1
Essential hypertension	3	0	3
Malignant hypertension	1	1	0
Coronary thrombosis	1	0	1
Angina pectoris	2	0	2
Varicose ulcers	1	0	1
Cerebral aneurysm	1	0	1
Mesenteric thrombosis	1	0	1
Total	31	1	30
Blood dyscrasia			
Pernicious anemia	4	0	4
Agranulocytosis	2	0	2
Thrombocytopenic purpura	1	0	1
Myeloid metaplasia	1	0	1
Total	8	0	8
Collagen			
Rheumatoid arthritis	5	1	4
Lupus erythematosis	1	0	1
Scleroderma	1	0	1
Total	7	1	6
Renal			
Glomerulonephritis	4	0	4
Pyelonephritis	1	0	1
Nephrosis	1	0	1
Hydronephrosis	2	0	2
Cystitis	1	0	1
Total	9	0	9
Acute infections			
Poliomyelitis	3	0	3
Fever of undetermined origin	3	1	2
Infectious mononucleosis	1	0	1
Pneumonia	1	0	1
Cholangitis	1	0	1
Total	9	1	8
Dermatological			
Pemphigus vulgaris	3	0	3
Neurodermatitis	1	0	1
Total	4	0	4
Postoperative			
Cholecystectomy	3	1	2
Choledochostomy	2	1	1
Ligation and stripping—varicose veins	1	0	1
Polypectomy	2	0	2
Partial gastrectomy	1	0	1
Herniorrhaphy	1	0	1
Total	10	2	8
Nervous system			
Cerebral vascular accident	2	0	2
Multiple sclerosis	2	0	2
Meningitis	1	0	1
Peripheral neuritis	1	0	1
Lumbar disc	1	0	1
Subdural hematoma	1	0	1
Epilepsy	1	0	1
Amyotrophic lateral sclerosis	1	0	1
Meniere's disease	1	0	1
Idiopathic cerebellar degeneration	1	0	1
Total	12	0	12

TABLE 1—*Continued*

Miscellaneous	Total cases	Pos.	Neg.
No final diagnosis	3	0	3
Fractures	3	0	3
Brucellosis	1	0	1
Moniliasis	1	0	1
Prostatitis	1	0	1
Gouty arthritis	1	0	1
Psychosis	2	0	2
Xanthomatosis	1	0	1
Aseptic necrosis	1	0	1
Breast abscess	1	0	1
Endometriosis	1	0	1
Osteoarthritis	1	1	0
Bronchiectasis	1	0	1
Hemochromatosis	1	0	1
Barbiturate poisoning	1	0	1
Arsenic poisoning	1	1	0
Total	21	2	19

No significant variation in results according to race, age, or sex could be observed in any of the diseases studied. The results of repeat tests done on 35 patients checked well in all cases with the original test.

It is difficult to present data accurately indicating the duplicability of CPI assays in the blood of human patients, since we did not permit this test to interfere in any way with the routine or extraordinary care of the individuals tested. However, duplicate assays of the same blood sample are invariably in good agreement and, further, the blood of animals under laboratory-controlled conditions is quite consistent in CPI activity, as demonstrated in the preceding paper. Some eight patients with nonmalignant disease were tested 2 or more times, and the results are tabulated in Table 2, which shows perhaps as consistent a picture as may be expected in view of the total lack of control of therapeutic measures, time and circumstances of blood withdrawal, etc.

TABLE 2

CONSISTENCY OF CARBOXYPEPTIDASE INHIBITOR (CPI) ACTIVITY IN BLOOD OF INDIVIDUALS WITH NONMALIGNANT DISEASE

Diagnosis	Date	CPI (units/ml whole blood)
Uremia	8/1	698
	8/8	704
Cholelithiasis	7/17	629
	7/21	566
Cirrhosis	8/14	612
	9/2	412
Hepatitis	7/22	748
	7/29	438
	8/6	522
	8/14	582
Sarcoidosis	7/16	652
	7/29	632
Peptic ulcer	7/10	470
	7/18	556
Ulcerative colitis	7/15	652
	8/15	462
Heart failure	7/23	708
	7/31	634

Consistency in the malignancy series is even more difficult to evaluate, because of the effect of the surgery in most of our cases. In at least some cases, surgery converted a positive to a negative test, or at least tended in that direction. Thus, one case of breast carcinoma gave a CPI titer of 116 units pre-operatively and 554 units 1 day after radical mastectomy and bilateral adrenalectomy; a patient with carcinoma of the colon showed a titer of 50 before surgery and of 188, 3 days after surgery; and a case of carcinoma of the colon showed a titer of 384 before and of 604 and 606, 10 and 13 days after surgery.

On the other hand, in many cases, surgery (bilateral adrenalectomy, ovariectomy, mastectomy, gastric resection, and others) either did not appreciably influence the CPI titer or even decreased it. The possibility must be considered, of course, that unknown metastases also existed in these cases.

DISCUSSION

The blood cell CPI test, like most of the other tests for malignant disease described to date, is not sufficiently specific to be considered diagnostic. All such tests have the common fault of becoming abnormal in most types of disease—i.e., they are tests more for disease in general than for cancer in particular. In this clinical survey, it is of interest to note that the average CPI titer in all diseases tested is lower than that in the normal group. The sole exception to this observation is seen in the pregnancy series, in which the titers were about equal to the normal although spread over a greater range. This is in contrast to most other tests for malignant neoplasia where pregnancy is commonly the cause of false positive results. Since pregnancy is a physiological state and not a pathological condition, it is not surprising that it fails to cause a change in the CPI titer. This suggests a nonhormonal etiology for the altered inhibitor concentration in disease.

Although only one disease process could be used as a basis for tabulation, some patients tested in this survey had dual diagnoses. Others, for whom only one disease had been diagnosed, might have been suffering from another pathological condition which was yet subclinical at the time the test was

done. Since all diseases tend to lower the CPI titer, it is entirely possible that multiple diseases might be cumulative in their effect on blood cell CPI. In addition, the possibility also exists that some of the diseases initially classified as nonmalignant may later be demonstrated to be cases of malignancy. There has, in fact, been one such change in our series of "false positive" liver disease cases since the attached chart was drawn up.

No effort was made to classify the malignant diseases into grade or stage or to group any of the nonmalignant diseases according to prognosis, duration, or severity of illness. Since the patients tested represent the extremes of these factors, results are bound to vary within each disease group. It is also possible that other conditions such as diet, activity, state of fatigue, and therapeutic procedures might greatly influence the CPI level. Since the normal group was rather uniformly controlled as to these conditions, whereas the clinical series was completely random, we are not able to evaluate this possibility.

The false positive results seen in a large percentage of patients with cirrhosis, tuberculosis, and diabetes are less important than the large number of false negative results in patients with cancer. The former, of course, are easy to diagnose by relatively simple laboratory and clinical procedures. The value of the test is greatly decreased, however, by its failure to become positive in histologically proved cases of cancer. Since the CPI test is nonspecific, its usefulness as a clinical diagnostic tool is limited. It perhaps may be of some value as a confirmatory test in conjunction with other tests in a screening program.

SUMMARY

The results of blood cell carboxypeptidase inhibitor (CPI) assays of the blood of 341 patients are reported. In normal persons and in cases of nonmalignant disease, the titer tends to be high, while in cases of malignant disease, the titer is greatly decreased. False positive results are observed chiefly in cirrhosis, pulmonary tuberculosis, and diabetes. False negative results are seen in 36 per cent of the malignant cases, chiefly carcinomas of the rectum and lung, malignant melanoma, lymphosarcoma, leukemia, and metastatic carcinoma of unknown primary site. All diseases tend to lower the CPI titer. The significance of this observation is discussed. The CPI test is not considered specific enough to be diagnostic for cancer.

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Studies on the Metabolism of Acetate-1-C^{14} in Tissues of Tumor-bearing Rats*

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In studies designed to test the oxidative activity of tumors *in vivo*, malonate was injected into rats bearing the Flexner-Jobling carcinoma, and succinate accumulation was found in the tumors and in most other tissues (3, 4). Following the establishment of the malonate block, acetate-1-C^{14} was injected, and the incorporation of label into the succinate pool was determined (5). In comparison with other tissues, the Flexner-Jobling carcinoma exhibited a very low incorporation of the label into the succinate pool. While most tissues rapidly transferred the tracer from a substance that is volatile in acidified solutions to compounds which are nonvolatile under the same conditions, the Flexner-Jobling tumor did not (5). The objectives of the present study have been (*a*) the extension of these findings to other tumors *in vivo* and (*b*) the investigation of the nature of the volatile compound or compounds which contained the bulk of the radioactivity in the tumor (1).

MATERIALS AND METHODS

Male rats, weighing 140–160 gm. and bearing tumors transplanted 8–10 days previously, were used in these studies. The tumors were four transplantable rat tumors: the Flexner-Jobling carcinoma, the Jensen sarcoma, the Walker 256 carcinosarcoma, and a transplantable lymphosarcoma.[1] In preparation of the tumor mince for transplantation, it was found useful to fit a 90-mesh stainless steel screen snugly into the hollow of a garlic press,[2] so that it rested on the perforated lower plate; the tumor mass was then placed on the screen, and the tissue was forced through the screen by pressure on the upper and lower arms of the press. The mince thus produced was diluted with saline solution, aspirated into a tuberculin syringe fitted with a 20-gauge needle, and injected into receptor rats. Sterile precautions were used throughout.

In the studies in which malonate was used, animals were given a single injection of 1.2 ml. of 1 M sodium malonate/100 gm body weight (3). One hour after the injection, 10^7 c.p.m. (counts per minute) labeled acetate[3] (10^7 cpm/mg) was injected intraperitoneally, and after 8 minutes the animals were sacrificed. In the intravenous series, 10^7 c.p.m. of the acetate-1-C^{14} was injected into the tail vein. Digital pressure was used to cause engorgement of the veins and was released when injection was begun.

The tissues were treated in the manner described previously (2). In the chromatographic analysis of the tissue samples, a glass manifold was substituted for the single column connected to the mixing flask (Chart 1). This manifold permitted simultaneous chromatography of eight tissue samples, and, accordingly, the mixing flask initially contained 1,600 ml. of distilled water (2). The glass columns containing the resin and samples were connected to the ground glass inner ends of the manifold (Chart 1). Effluents from these columns were collected in small test tubes (16 × 100 mm.) placed under the columns; at selected time intervals, the test tubes were replaced by empty tubes. Pressure on the system was maintained by the height of the reservoir (a separatory funnel); the volumes collected per tube averaged about 2.0 ml. Emergence of known peaks does not occur in exactly the same fractions from each column—i.e., peaks generally tend to emerge earlier in the course of a run from columns connected to the center of the manifold than those connected to the sides. In addition to the possibility of collection of eluates from a number of samples simultaneously, the procedure has the advantage of availability to laboratories that are not equipped with automatic fraction collectors.

A further alteration in technic was the use of acetic acid to elute compounds from a column of Dowex-1 in the acetate form. With the previous system employed (2), acids of the citric acid cycle were well separated, but lactic, glutamic, aspartic, and acetic acids were not. As is shown in Chart 2, separation of these acids has been possible with the acetic acid-Dowex-1 acetate system. The concentration of acetic acid in the reservoir was 4 N. This separation permitted identification of

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[1] Animals bearing Flexner-Jobling, Jensen, and Walker tumors were furnished by Dr. G. A. LePage. The lymphosarcoma was obtained from Dr. I. R. Sherman. Subsequent transplantations of the tumors were carried out in this laboratory by Mrs. R. K. Busch.

[2] Use of the garlic press was suggested by Dr. G. A. LePage.

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[3] Two samples of acetate-1-C^{14} were used in these studies, one prepared in collaboration with Dr. C. Heidelberger at the McArdle Memorial Laboratory and the other obtained from the Tracerlab, Inc. Volatility of the radioactivity was 99.8 per cent in acidified solutions. In this paper, the term "counts" indicates the quantity of radioactivity recorded on the scalers available. On the instruments used, 10^7 c.p.m. equals 0.01 mc.

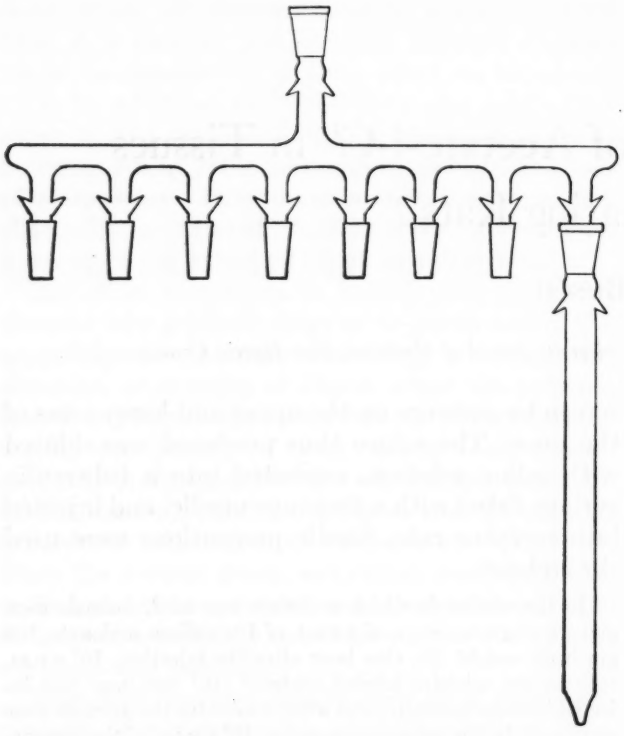

CHART 1.—Manifold used for simultaneous chromatography of multiple tissue samples. One glass column is shown connected to the manifold. The standard taper joints are 14/35, the whole manifold is 14 inches wide, and the connections for the glass columns are spaced $1\frac{3}{4}$ inches apart. The outer ground glass end above is connected to the outflow from the mixing bowl.

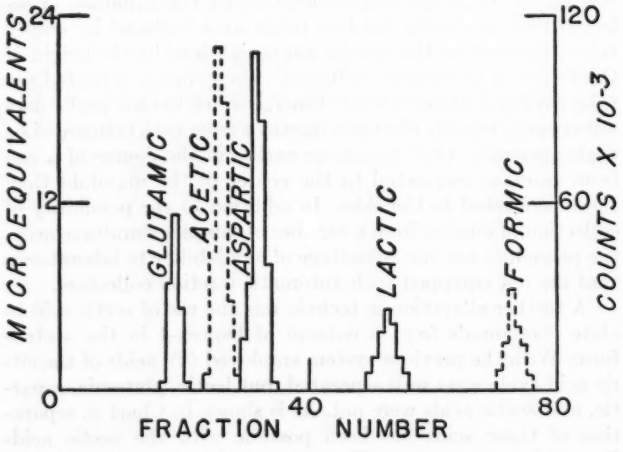

CHART 2.—Anion exchange chromatogram of a mixed sample of glutamic, aspartic, lactic, acetic-1-C^{14}, and formic-C^{14} acids. Acetic acid was the eluting agent and Dowex-1 acetate was the stationary phase. Glutamic and aspartic acids were determined by titration, and lactic by colorimetric analysis. The broken lines indicate radioactivity in acetic acid-1-C^{14} and formic acid-C^{14}. The concentration of acetic acid was 0.5 N at fraction 20, 1.3 N at fraction 40, 3.0 N at fraction 60, and 4.0 N at fraction 80.

the compound containing the radioactivity as well as the quantitative determination of the radioactivity present.

RESULTS

Malonate-treated Animals

The incorporation of label from acetate-1-C^{14} into the succinate pool of a number of tumors was the initial point tested. In these studies, a nonlethal dose of malonate was used—i.e., a single injection of 1.2 ml. of 1 M sodium malonate/100 gm body weight. Table 1 shows the specific activity of the succinate isolated from samples of the tumors. There was a uniformly lower specific activity of the succinate of the tumors as compared to that of other tissues. In previous studies, samples from lung, heart, and skeletal muscle have given results similar to those of the kidney, liver, and spleen.

TABLE 1

RADIOACTIVITY INCORPORATED INTO SUCCINATE OF TISSUES OF MALONATE-TREATED RATS

Tissue	No. samples	Specific activity succinate (cpm/μM)	Av. deviation	Per cent total counts in succinate
Kidney	6	2,609(1,110–4,370)	940	29.9
Spleen	2	1,910(1,610–2,210)	300	7.2
Liver	1	1,350		8.8
Jensen sarcoma	3	94(0–282)	100	2.3
Walker 256 carcinosarcoma	2	173(136–211)	37	5.5
Lymphosarcoma	2	80(68–92)	12	3.0

As an approximation of the conversion of acetate to other compounds, the percentage of total radioactivity in compounds nonvolatile in acid medium was determined. The average counts/gm tissue in this series were 11,200 for the tumor, 4,900 for brain, 11,000 for testis, and 37,500 for kidney. In the malonate series, most tissues converted from 60 to 100 per cent of the total radioactivity present into nonvolatile compounds (Table 2). The average conversion for the tumors was about 32 per cent, and, as can be seen in Table 2, the results for the different tumors were not statistically different.

The percentage of the total label in the various peaks of the acid profile was determined. In the kidney, 30 per cent of the total radioactivity was in the succinate peak, while in other tissues succinate accounted for about 10 per cent of the total radioactivity present (5). In the tumors, only 3–5 per cent of the total radioactivity was in succinate. Of the other peaks of the acid profile studied, peak 2 was found to contain large percentages of the total activity (5). In the spleen, as much as 55 per cent of the total activity was in this peak, while,

in other tissues, 25–35 per cent of the total radioactivity was in this peak (Table 3). In previous studies (5) on samples prepared from spleens of tumor-bearing animals, the chief component of this peak was identified as glutamate by anion exchange chromatography, by paper chromatography, and by cation exchange chromatography. Inasmuch as the acetic acid-Dowex-1 acetate system cleanly separated glutamate from aspartate and other substances (Chart 2), correlation of radioactivity with titration from added carrier glutamate seemed sufficient to establish the identity of the radioactive compound as glutamate (Table 4). Supporting evidence for this point included the position of emergence of the compound on the original anion exchange chromatogram and the equivalence of ninhydrin color and titration. The percentage of the total radioactivity accounted for in succinate and glutamate combined was approximately 12 per cent for the tumors and 25–65 per cent for the other tissues.

TABLE 2

PERCENTAGES OF RADIOACTIVITY TRANSFERRED TO NONVOLATILE COMPOUNDS IN TISSUES OF MALONATE-TREATED RATS

Values are percentage of total in tissues

Tissue	No. samples	Per cent nonvolatile	Av. deviation
Spleen	2	107(100–113)	7
Brain	2	105 (77–123)	18
Liver	2	94 (90–97)	4
Kidney	6	92 (78–100)	9
Testis	7	56 (41–74.5)	10
Jensen sarcoma	3	32 (20–43)	8
Walker 256 carcinosarcoma	2	37 (28–47)	10
Lymphosarcoma	2	25 (12–38)	13

TABLE 3

PERCENTAGE OF TOTAL RADIOACTIVITY IN GLUTAMATE OF TISSUES OF MALONATE-TREATED RATS

Values are percentage of total in tissue

Tissue	No. samples	Per cent in glutamate	Av. deviation
Spleen	1	55	
Kidney	6	37(8–77)	18
Liver	2	35(26–45)	10
Brain	3	30(16–48)	12
Testis	6	24(14–32)	7
Jensen sarcoma	3	9(3–13)	4
Walker 256 carcinosarcoma	2	14(13–14)	1
Lymphosarcoma	1	3	

TABLE 4

CORRELATION OF ISOTOPE FROM PEAK 2 WITH TITRATION OF CARRIER GLUTAMIC ACID

Sample	Fraction no.	μ Eq.	Total counts	cpm/μ Eq.
Kidney, J_4	15	5.5	5,000	902
	16	22.6	19,600	867
	17	11.2	9,850	895
Spleen, C_1	26	1.9	1,980	1,040
	27	16.4	17,420	1,060
	28	28.6	30,600	1,070
	29	7.6	8,070	1,065

UNTREATED ANIMALS

Intraperitoneal series.—One important objection to previous studies (5) was the possibility that results obtained in tumors might have reflected a peripheral hypotension accompanying malonate treatment.[4] Accordingly, it was thought desirable to study the indices of acetate utilization available in tumors of untreated animals (cf. 4)—namely, percentage of total radioactivity converted to nonvolatile compounds and percentage of total radioactivity in compounds of the acid profile. In the initial series, acetate-1-C^{14} was injected intraperitoneally, and, 8 minutes later, the animals were sacrificed; the omission of malonate injection was the only change from preceding experiments. In the six experiments of this series, the total counts per gram of tissue were 13,000 for the tumors, 4,600 for the brain, 5,500 for the testis, and 45,500 for the kidneys. The average nonvolatile radioactivity in the tumors was 44 per cent of the total radioactivity, compared to 67 per cent for the testis and 94 per cent for the kidney. Little change was noted in the results for other tissues studied. In the blood, the nonvolatile radioactivity comprised 42 per cent of the total radioactivity. The average percentage of radioactivity found in glutamate was 19 per cent for the tumors, 63 per cent for the kidney, 30 per cent for the testis, 34 per cent for the brain, and 54 per cent for the spleen.

Intravenous series.—Injection of tracers intraperitoneally has a number of disadvantages in the study of metabolism of substances which are turned over at rapid rates. Thus, it is difficult to estimate the half-time of absorption of acetate from the blood by the tissues when the acetate in the blood is being continuously replenished from the peritoneal cavity.[5] Secondly, there is the possibility of large-scale metabolism of the precursor as it enters the portal circulation and passes through the capillary bed of the liver. When feasible, experiments in which the tracer was injected intravenously seemed preferable, since in this type of study there is rapid diffusion of the labeled com-

[4] Other signs of malonate toxicity are irritation at the injection site, dehydration, reduced peripheral circulation, and death from sufficiently large doses.

[5] The half-time of absorption of acetate-1-C^{14} has been shown by Hutchens (6) to be about 1 minute.

pound from the blood into the tissues and, with shorter time periods, there is less chance of the transportation of metabolic products of the precursor from tissue to tissue. In the following experiments, acetate-1-C^{14} was injected intravenously, and after 3 minutes the animals were decapitated

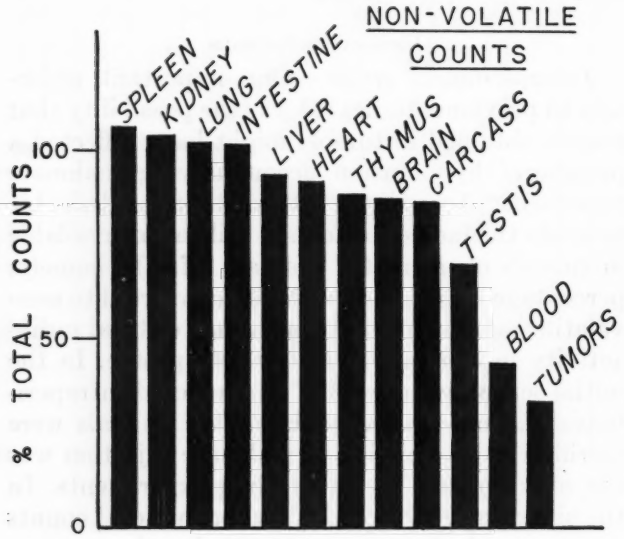

CHART 3.—Nonvolatile radioactivity in tissues 3 minutes after the injection of acetate-1-C^{14}. Values are averages for seven samples each of tumor, testis, and kidney; three samples of blood; two samples each of liver, spleen, and intestine; and one each of the other tissues. The average deviation of results from the values shown was 7.5 per cent for tumors, 5.5 per cent for the testis, 9.5 per cent for the kidney, 3 per cent for the blood, 3 per cent for the liver, 10 per cent for the spleen, and 7 per cent for the intestine. Of the seven tumor samples, there were two each of the Walker 256 carcinosarcoma, Jensen sarcoma and lymphosarcoma, and one of the Flexner-Jobling carcinoma. In these experiments, the animals were not injected with malonate.

and the tissues excised; the tissues were then treated as previously described (2). As Chart 3 indicates, in nontumor tissues of the tumor-bearing rat, the conversion of the radioactivity to nonvolatile compounds was largely complete by this time, and, in all tissues other than tumor tissues studied, the percentage of radioactivity converted to nonvolatile compounds was greater than that in the blood. In the six results for tumors, the conversion to nonvolatile compounds was lower than in other tissues. Moreover, the percentage of radioactivity found in glutamate in the tumors was very nearly that of the blood (Chart 4) and was much lower than in the other tissues studied.

IDENTIFICATION OF THE VOLATILE COMPOUND

In the tumors, the largest fraction of the radioactivity remained in a compound volatile from acid solution; this compound was shown to be acetate by a number of methods. *Duclaux series* on fractions from the chromatogram which contained the volatile radioactivity of a sample from a Walker tumor gave the following results for titration: 10.1, 10.3, 10.6, and 11.0 per cent in the first four fractions; and, for radioactivity, 8.9, 9.1, 9.4, and 9.7 per cent in these same fractions. Another series gave 7.0, 8.2, and 9.4 per cent for radioactivity of the first three fractions and 7.3, 8.4, and 8.9 per cent for titrations in the same fractions. The *p-bromphenacyl ester* was prepared (7) from neutralized perchloric acid supernatant solution of two tumor samples, and the results are shown in Table 5, J_5Tu and J_4Tu. These derivatives were recrystallized to constant specific activity. In other experiments, the chromatographic fractions containing the volatile radioactivity were neutralized, and the *p*-bromphenacyl ester was formed with results given in the table for the F_1Tu and W_5Tu samples. Total radioactivity calculated from the cpm/mg derivative approximated total radioactivity volatile from the original solutions.

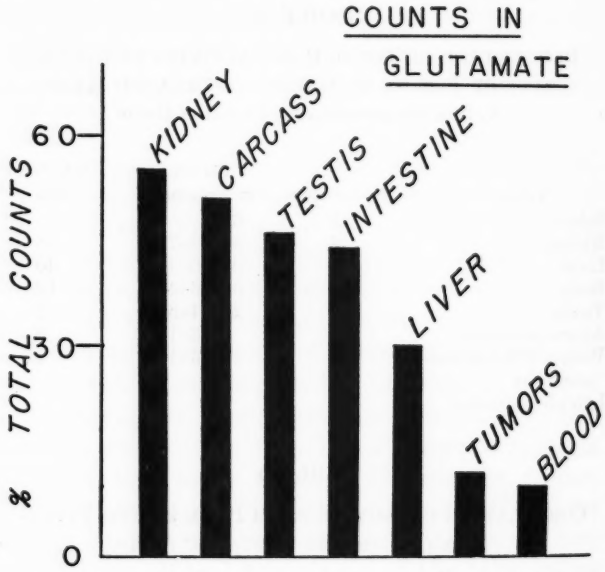

CHART 4.—Percentage of total radioactivity in glutamate in tissues 3 minutes after the intravenous injection of acetate-1-C^{14}. There are seven samples of testis, six each of tumor and kidney, three of blood and two of liver and intestine. One sample each of carcass, heart, lung, brain, and thymus were also studied, and values ranged from 46 to 49 per cent conversion to glutamate. In one sample of spleen, 61.8 per cent of the total counts were in glutamate. Average deviations were 3.1 per cent for the tumors, 9.3 per cent for the testis, 12 per cent for the kidney, 2.3 per cent for the blood, 1 per cent for the liver, and 6 per cent for the intestine. Malonate treatment was omitted in these experiments.

Under the conditions of determination of the volatile radioactivity (5), 99.8 per cent of the radioactivity of the acetate-1-C^{14} injected were volatile. Following the demonstration of the chromatographic position of the acetic acid (Chart 2),

it was possible to determine the relative percentages of the isotope in glutamic and acetic acids directly. In a number of samples in which the total radioactivity was determined, the ratio of isotope in acetate to isotope in glutamate within the tumors was 2.7:1, with a variation of 1.1–4.8 in a series of seven samples. The values for the same ratio in the testis ranged from 0.0 to 0.25, while for most other tissues the value approached 0.0, since there was virtually no acetate present. In one sample of the blood, the ratio was 6.0 as compared to 3.1 for the corresponding tumor; this value again indicates the parallelism between the tumor and the blood. While these data are evidence for the persistence of acetate in tumors as such, it is possible that other volatile compounds may contain small quantities of radioactivity.

TABLE 5

PERSISTENCE OF ISOTOPE IN THE *p*-BROMPHENACYL ESTER OF ACETIC ACID

The derivative was prepared from the sample containing volatile radioactivity in the presence of carrier acetate; recrystallization to constant specific activity was carried out.

Sample	Source	Stage of purification	Melting point	cpm/mg
J_5Tu	Neutral PCA*	Original	83	18.1
		Once recrystallized	84–85	19.2
		Twice recrystallized	86–87	22.1
J_4Tu	Neutral PCA	Original	82–83	33.5
		Once recrystallized	84–85	43.8
		Twice recrystallized	84–85	44.0
F_1Tu	Chromatographic fractions	Original	81–83	76
		Once recrystallized	83–84	106
		Twice recrystallized	84–85	109
W_5Tu	Chromatographic fractions	Original	77–80	29.9
		Once recrystallized	84–85	34.4
		Twice recrystallized	85–86	36.1

* Neutralized perchloric acid solution.

DISCUSSION

Three types of experimental data relevant to the problem of acetate metabolism in tumors *in vivo* are presented. (*a*) In terms of radioactivity incorporated into succinate, either as percentage of the total activity, as specific activity of the isolated succinate, or as radioactivity in succinate per gram of tissue, the tumors incorporated much less activity than other tissues; (*b*) the persistence of the label in acetate, at a level approximately equal to that of the blood, demonstrates diminished ability of the tumors to convert acetate to other substances, while in a number of tissues, conversion of acetate to other substances proceeds so rapidly that volatile radioactivity could not be found; (*c*) the conversion of acetate to glutamate in tissues other than tumors accounted for 25–50 per cent of the total radioactivity present in the tissue. With regard to the label in glutamate, the data suggest an equilibration of the blood and the tumor.

Of the other tissues of the tumor-bearing rat, the testis was most like the tumor in persistence of volatile radioactivity. Even in this case, very significant differences from values obtained for tumors were found in the percentage of total isotope incorporated into glutamate and other nonvolatile compounds. Moreover, failure to find appreciable radioactivity in the acetate peak (see ratio of isotope in acetate to isotope in glutamate) suggests the possibility that the volatile radioactivity in the testis might be in a substance other than acetate. The results for the brain were very different from those of the tumors. It is of interest that the tumors contained more radioactivity per gram of tissue than did either brain or testis.

While these and other experiments (8, 9) show that the failure of the Flexner-Jobling tumor to convert acetate to other compounds is not unique to that tumor, it is not possible to generalize concerning all tumors, particularly those which represent mixed cell types.

These findings would tend to indicate that utilization of the acetate molecule, a function which is extensively available to most other tissues, is either very markedly diminished in or totally deleted from these tumors.

SUMMARY

1. In further development of chromatographic technics, an apparatus was constructed for simultaneous analysis of eight tissue samples. Chromatographic separation of a mixture of acetic, glutamic, aspartic, lactic, and formic acids was achieved in a system in which acetic acid was the eluting agent and Dowex-1 in the acetate form was the stationary phase.

2. With the above technics, further studies on the metabolism of acetate-1-C^{14} in tumor-bearing animals were carried out. In a series of tumors of malonate-treated animals, the specific activity of the succinate accumulated was 1/20th that of the liver, kidney, and spleen, 8 minutes after the injection of the tracer.

3. While most tissues of tumor-bearing animals converted 70–100 per cent of the total radioactivity reaching the tissue into nonvolatile compounds, the bulk of the radioactivity in the tumors persisted as acetate.

4. When acetate-1-C^{14} was injected into ani-

mals that were untreated with malonate, 30–60 per cent of the total radioactivity was found in glutamate in tissues other than tumors, while, in the tumors and blood, about 10 per cent of the total radioactivity was found in this substance 3 minutes after the injection of the tracer.

5. In the untreated animals, 8 minutes after intraperitoneal injection of acetate-1-C^{14} and 3 minutes after intravenous injection of the tracer, the percentage of isotope transferred to other compounds was approximately the same as that in the malonate series.

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Phospholipid and Protein-bound Phosphorus Synthesis in the Rabbit Papilloma*

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The Shope virus characteristically produces papillomatosis in the rabbit. The infection has been observed to terminate either in recovery or in metastatic squamous-cell carcinoma (9, 19). Chemically, the papilloma contains 27 per cent water (20). The protein has been classified as a pseudokeratin, and amino acid analyses indicated that at least eighteen are represented in the protein of the Shope papilloma virus (15, 20). The virus is 57 per cent hydrolyzed by pepsin at a pH of 1.8 and 87 per cent by trypsin at a pH of 7.9 (20). In the present investigation an attempt has been made to study, with the aid of radioactive phosphorus, the metabolism of phospholipid phosphorus and protein-bound phosphorus (nucleo- and phosphoprotein) in the papilloma as compared to normal skin of the rabbit. A determination of the distribution of lipids and the phosphorus fractions has been made in these tissues.

MATERIALS AND METHODS

Adult female rabbits (1.5–2.1 kg.) of the Dutch belted variety were fed stock diet and water ad libitum. The animals were divided into two groups—normal rabbits and those inoculated with the Shope papilloma virus. The virus preparation and the inoculation were carried out after the method of Fischer and Green (7). The papilloma appeared in about 3 weeks and grew rapidly in approximately 90 per cent of the cases. Only those animals with actively growing papillomas were used. Two to 9 months after the virus inoculation, the animals were injected intraperitoneally with 1 ml. of physiological saline containing 150–200 microcuries of P^{32} as Na_2HPO_4. Ten hours after the injection the animals were sacrificed by chloroform anesthesia and heart puncture. The tissues were immediately removed from the animal. The subcutaneous fat and connective tissue and the hair were removed, as far as possible, with scissors. The samples of skin and papilloma were then weighed (1–10 gm.) and covered with cold 10 per cent solution of trichloroacetic acid (TCA) containing 0.5 M $MgCl_2$ (11), minced with scissors, and ground in previously chilled mortars. The resulting paste was washed quantitatively with cold TCA solution into centrifuge tubes, diluted to volume, and allowed to stand for at least 30 minutes in the cold (−10° C.), and then centrifuged and filtered in the cold. The total acid-soluble P was determined on the filtrate (8). The lipids were extracted from the TCA-insoluble residue with alcohol and alcohol-ether and purified with chloroform (2). On aliquots of the chloroform solution, the weight of the lipids, their radioactivity (2), and their phosphorus content (8) were determined. All radioactivity measurements were made by a Geiger-Müeller counter (Superscaler, Tracerlab). After the solvent extraction (TCA, alcohol-ether), the liver residue was digested for 3 hours at 90° C. in 1 N NaOH to determine total protein-bound phosphorus (17). This fraction contains both the nucleoprotein and phosphoprotein P (10). Phosphoprotein is only a minor component of animal tissue (10), but Johnson and Albert have shown that the "phosphoprotein" fraction incorporated P^{32} at a very high rate (10). On aliquots, the total phosphorus (8) and radioactivity were determined. From these results and the radioactivity measurements, the specific activity,[1] relative specific activity,[2] and relative radioactivity[3] of the phospholipids and nucleoproteins were calculated.

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[1] The specific activity is the ratio of radioactivity (in relative radioactivity units, per cent of dose of P^{32} injected × 100) to the phosphorus (in mg.).

[2] The relative specific activity is the ratio of the specific activities of the phospholipid or nucleoprotein to that of the acid-soluble phosphorus.

[3] The relative radioactivity is the ratio of the radioactivity (in relative radioactivity units, per cent of dose of P^{32} injected × 100) to the specific activity of the acid-soluble phosphorus.

Under the conditions of our experiments, an increase in the formation of phospholipid and protein-bound phosphorus fractions may reasonably be assumed when higher values for specific and relative activities and relative radioactivities are found. Various factors, such as differences in the rates of absorption and in the equilibrium between intra- and extra-cellular inorganic phosphorus with different specific activities, may conceivably produce changes in the specific activity of the inorganic fraction. Comparison of the relative specific activities or relative radioactivities should provide a means of reducing these causes of error.

TABLE 1

THE AVERAGE VALUES OF PHOSPHOLIPID P AND PROTEIN-BOUND P SYNTHESIS IN THE RABBIT PAPILLOMA AND NORMAL SKIN*

		Phospholipid P/gm wet tissue			Protein-bound P/gm wet tissue			R.S.A. phospholipid P†
	No. animals	Specific activity	Relative specific activity	Relative radioactivity	Specific activity	Relative specific activity	Relative radioactivity	R.S.A. protein-bound P
Skin	10	0.20±0.16	0.060±0.032	0.007±0.006	0.26±0.16	0.086±0.044	0.042±0.025	0.804±0.378
Papilloma	16	2.40±1.93	0.292±0.093	0.046±0.016	2.17±1.72	0.259±0.073	0.221±0.056	1.129±0.130
Difference between means	*t*	3.461	7.265	2.257	3.372	6.370	9.456	3.021
		P<0.01	P<0.01	P<0.05	P<0.01	P<0.01	P<0.01	P<0.01

* The figures preceded by the ± sign indicate the standard deviations. *t* is the test of significance applied to the difference between the means. P is the probability for chance occurrence of this difference.

† R.S.A. = Relative Specific Activity.

TABLE 2

THE AVERAGE VALUES OF DISTRIBUTION OF PHOSPHORUS AND LIPID IN THE RABBIT PAPILLOMA AND NORMAL SKIN*

		Mg/gm wet tissue			
	No. animals	Total lipids	Total acid-soluble P	Total phospholipid P	Total protein-bound P
Skin	10	97.4±33.6	0.116±0.045	0.104±0.032	0.468±0.084
Papilloma	16	60.8±12.5	0.368±0.063	0.161±0.032	0.873±0.100
Difference between means	*t*	3.775	11.485	4.053	10.146
		P<0.01	P<0.01	P<0.01	P<0.01

* The figures preceded by the ± sign indicate the standard deviations. *t* is the test of significance applied to the difference between the means. P is the probability for chance occurrence of this difference.

RESULTS AND DISCUSSION

The data obtained from the present investigation concerning the metabolism of phospholipid and protein-bound P as well as the total lipid and phosphorus distribution in the two groups of rabbits, are reported in Tables 1 and 2. To evaluate the statistical significance of the results, the *t* test of significance (4) was applied to the difference between the means of the results from each group.

The data in Table 1 indicate that the specific activities, relative specific activities, and relative radioactivities of both the phospholipid and protein-bound P fractions of the papilloma were statistically increased as compared to those of skin, its tissue of origin. This would indicate there is an increase in the incorporation of the P^{32} into both phospholipid and protein-bound P fractions in the papilloma. There is an increased concentration of both phospholipid and protein-bound P in the papilloma, since the relative radioactivity is a measure of the newly formed molecules (1). However, if the ratio of the relative specific activity of phospholipid P to that of the relative specific activity of protein-bound P is compared in the skin and the papilloma, it is noted that the ratio is greatest in the papilloma. This would indicate that phospholipid synthesis, as evidenced by the P^{32} incorporation, is greater in the papilloma than is the protein-bound P synthesis. If the synthesis of both fractions were stimulated equally in the papilloma, although P^{32} incorporation in the papilloma would be greater, the ratio of the relative specific activity of phospholipids to that of protein-bound P would be the same in the papilloma as in the skin.

Campbell and Kosterlitz (3) have demonstrated in the rat that the ratio of liver protein nitrogen to phospholipid phosphorus remains approximately constant on various levels of dietary proteins. Phospholipid synthesis appears to be related to that of nucleoprotein, since a constant ratio has been observed under various dietary regimes (5). In the present studies, the ratio of phospholipid to protein-bound phosphorus synthesis was considerably increased in the papilloma as compared to normal skin. The papilloma contains a smaller amount of total lipids than the skin, but a larger amount of acid-soluble and nucleoprotein phosphorus.

The Shope virus papilloma has been shown to have an increased rate of metabolism when compared to other tissues and a higher anaerobic and a moderately higher aerobic glycolysis accompanied by a respiratory quotient below unity (6, 14). The increased synthesis of phospholipids and nucleoproteins by the papilloma is in accordance with the results of other investigators, who have shown that there is an increased turnover in these fractions in other tumors (12, 13, 15, 16, 18).

SUMMARY

The phospholipid P and protein-bound P synthesis in the rabbit papilloma (virus, Shope) and skin have been measured with the aid of radioactive phosphorus. If the specific activity, relative specific activity, or relative radioactivity values are taken as a measure of the synthesis of these fractions, there is a significant increase in phospholipid P and protein-bound P in the papilloma ($P < 0.01$) when compared to normal skin.

The papilloma contained less lipid and more phosphorus in the acid-soluble and protein-bound fraction than did the skin.

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Incorporation of Labeled Methionine *in Vitro* by Tissues of C3H Mice*†

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Mider (15) and others (20) have observed that malignant tumors are characterized by a high priority for amino acids, and, in the absence of an adequate diet, they can grow at the expense of normal tissues. Among various possibilities which might explain this phenomena is that of a relative increase in the activity of the enzymes which control the rate of protein synthesis.

In recent years several investigators have studied the uptake of labeled amino acids by the proteins of tissue preparations (3, 14, 17, 21). Considerable evidence has been accumulated which indicates that this labeling of protein, under suitably controlled conditions, represents an enzymic synthesis of peptide bonds. Thus, the protein-labeling reaction may afford an index of the overall activity of the enzymes involved in the anabolic phase of protein metabolism, and it becomes of interest to investigate a possible relationship between this reaction and neoplastic processes. This report concerns an investigation of the rate of protein-labeling by the tissues of C3H mice, which are characterized by a high incidence of spontaneous tumors of the mammary gland (1, 2). It has been found that the appearance of the tumors is associated with a marked increase in the activity of the protein-labeling enzymes. Hence the effect of several factors known to be related to the production of these tumors has been studied.

MATERIALS AND METHODS

Adult mice of Bittner's C3H subline (1) were used throughout this study. The majority were received from the Jackson Memorial Laboratory at 4–5 weeks of age, the rest being first-generation offspring of our colony. As controls C3H females free of the maternally transmitted milk factor (2) were employed. These animals, referred to hereafter as "foster-nursed," do not develop tumors. They had been started from C3H ova implanted into a C57B1/Jax female. The animals used were seventh or eighth generation offspring from this single outstrain contact, which served to eliminate the maternally transmitted milk agent.[1] A second group of controls consisted of C3H males, which have been shown to be resistant to mammary tumors unless given female hormone (5, 12). All animals were sacrificed either by decapitation or by breaking the neck —in either case death was essentially instantaneous.

Mammary tissue was taken from recent mothers, whose young had been weaned within the previous week. It was obtained, after removal of the hair with a dry razor, by stripping the skin containing the gland from the underlying subcutaneous layer. Histological examination of similar preparations showed them to be diluted considerably with skin tissue, containing of the order of 50 per cent mammary tissue. In nonlactating females as well as in males, there is a practical absence of distinctive mammary tissue.[2] In these animals skin was chosen as the tissue which most closely resembles the cells from which the tumors develop. The sections were cut from the region surrounding the nipple and the rudimentary duct. From 2–5 pieces of tissue from a single animal were used in each determination. In the experiments on the *in vitro* effects of hormones on methionine uptake, corresponding sections from the same animal were used for the control value. For the measurements with liver and tumor tissue slices were prepared free hand, one or two slices being used in each flask.

L-methionine containing S^{35}, obtained from Dr. D. L. Tabern of the Abbott Laboratories, was employed as the labeled addendum throughout this work. A chromatogram supplied with the methionine showed it to be free of other substances containing S^{35}.

The method used to determine protein-labeling consists of incubation of the tissue with the labeled amino acid in a Krebs-Ringer bicarbonate solution containing 7.8×10^{-3} M succinate. The methods used have been described in detail in a previous communication (13). The only modifications introduced in this work were the use of L- instead of DL-methionine, and the centrifugation of the tissue immediately after the trichloroacetic acid was added to stop the reaction. The tissue was then homogenized with fresh trichloroacetic acid. With this modification it was possible to increase the methionine concentration to 1 μM/ml without increasing the zero time value. Preliminary experiments showed that the extent of labeling remains constant when the substrate level is changed from 0.5 to 1.0 μM methionine/ml. Hence, in the measurements reported here the enzymes were functioning at their maximum rates under the conditions used.

Unless otherwise specified, all data in the tables are ex-

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† Portions of this paper were presented before the American Society for Biological Chemists in Chicago, April, 1953 (12).

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[2] The authors are deeply indebted to Dr. Arthur J. Gatz for the preparation and interpretation of the histological sections.

pressed as μM S^{35} incorporated into protein as methionine per kilogram wet weight per hour. The incubation times were either 1 or 2 hours. The rate of incorporation was found to be strictly linear for 2 hours with skin. Simpson and Tarver reported similar results with liver slices (19). It is assumed that this linearity holds for the other tissues investigated.

RESULTS AND DISCUSSION

Comparison of tumor and normal tissues.—A series of measurements were made on various normal tissues to serve as a basis for comparison to the activity of the tumor tissue in labeling protein (see Table 1). A value for liver is included for the purpose of orientation with the work of other laboratories, since liver has been most thoroughly studied with respect to amino acid incorporation (14, 17, 19, 22). Pituitary tissue is considerably more active than liver in this regard; this is in agreement with previous studies made on rats (13). Skin tissue is seen to be quite active in protein-labeling; it is about three times as active as liver. The value for mammary tissue is higher than that for the skin, and it is apparent that it must be minimal, since the tissue is diluted with less active epidermal cells. If one assumes that one-half of the tissue is actually mammary tissue, the value would be raised to 113. On the other hand, the tumor slices showed an uptake of the label which is significantly greater than for any other tissue studied. The activity of the tumors would appear to be far higher than that of mammary tissue, even when allowance is made for the error in the determinations on mammary tissue mentioned above.

TABLE 1

INCORPORATION OF METHIONINE BY TISSUES

TISSUE*	METHIONINE INCORPORATION† μM/kg/hr
Liver (6)	27 ± 3
Pituitary (female) (12)	164 ± 15
Skin (26)	81 ± 3
Mammary (10)	97 ± 4
Tumor (8)	215 ± 14

* Number of measurements given in parentheses.
† Plus or minus the standard error of the mean.

Since the appearance of the tumor results in a marked increase in the rate of protein-labeling, it was of interest to investigate the possibility that a tendency for a higher activity of these enzymes may occur prior to tumor development. Skin was selected as the tissue, available in the three groups of animals, which most closely resembles that from which the tumors originate. The data obtained are presented in Table 2. No significant difference among the three groups was observed.

It is interesting to note that other workers (22) have observed an increase in the rate of protein-labeling in *p*-dimethylaminoazobenzene-induced hepatoma over that found in liver. In the present paper a similar increase is observed in the spontaneous mammary tumors of C3H mice. This is apparently not a general property of all tumors, however. Kit and Greenberg (10) have found no increase in the labeling-rate in Gardner lymphosarcoma cells over related normal tissues such as spleen and lymph nodes. The work of Zamecnik *et al.* (22) indicated a pre-tumor effect of the azo dye on the liver cells; the slices prepared from normal liver from animals bearing hepatomas showed a greater rate of labeling than those from untreated controls. In our studies on a spontaneous tumor, no difference could be detected between tissues taken from the C3H females, in which tumors would have developed if the animals had been permitted to live, and from animals lacking the milk factor or the female hormone, in which no tumors would develop. In two experiments skin was taken from females bearing a tumor elsewhere, and the values obtained showed no increase over the normal range. Thus it seems that no gross change in the enzymes under study can be detected prior to the appearance of the tumors.

TABLE 2

INCORPORATION OF METHIONINE BY SKIN*

GROUP	METHIONINE INCORPORATION μM/kg/hr
C3H female (5)	90 ± 7
C3H male (16)	81 ± 4
Foster-nursed C3H (6)	82 ± 4

* See footnotes to Table 1.

In vitro effects of hormones.—The production of tumors in the male mice by the administration of estrogenic compounds has been described by Lacassagne (11) and by Burns and Schenken (5). Hence, it was of interest to determine if the increased activity of the protein-labeling enzymes could be produced by estrogenic compounds *in vitro*. Since estrone and testosterone have been used therapeutically in cases of breast cancer (8, 9) the effect of these substances on the tumor has also been observed.

In all cases the hormone was added to the flask as an aliquot of an alcoholic solution, followed by evaporation to dryness before adding the standard medium. In some of the experiments the tissue was pre-incubated in the medium containing the hormone for up to 1 hour before the labeled methionine was added; the controls were handled in an identical manner. It is interesting that this pre-incubation at room temperature did not ap-

pear to effect the protein-labeling, in view of the observations of Simpson and Tarver (19) and Rutman (18) that a marked decrease in the incorporation of methionine by liver tissue occurred when the slices were stored in ice-cold Ringer's for an hour or more prior to incubation.

As can be seen from Table 3, estrone *in vitro* has

TABLE 3

EFFECT OF HORMONES *in Vitro**

Tissue	Hormone†	Methionine incorporation μM/kg/hr
C3H male skin	None (12)	81 ± 5
	Estrone (12)	79 ± 4
C3H female, mammary	None (10)	97 ± 8
	Estrone (10)	97 ± 4
C3H female, tumor	None (8)	215 ± 14
	Estrone (10)	207 ± 19
	Testosterone (3)	246 ± 22

* See footnotes to Table 1.

† One to two micrograms per milliliter.

no effect on the incorporation of methionine into protein by skin, mammary tissue, or tumor slices. Similarly, no significant effect of testosterone on the tumor was noticed.

Effect of estrone in vivo.—In spite of the negative results with estrone added *in vitro*, it is apparent that the administration of estrone must eventually affect the protein-labeling enzymes, since continued administration will result in the appearance of tumors (16). Hence, a series of C3H males were given subcutaneous injections of 22 μg estrone/day, dissolved in 0.1 ml. of Mazola, 5 days a week. The animals were sacrificed prior to tumor development since our interest was in a possible pre-tumor increase in the activity of the enzymes under study.

As is evident from Table 4, no significant effect on the uptake of labeled methionine by the skin tissue of the male is obtained by this procedure. These results would appear to indicate that no pre-cancerous change occurs in this process. However, the possibility remains that the effect of the hormone is on one or perhaps a few single cells. In such a case the methods available at present could not detect it.

Measurements with pituitary tissue.—Since the tissue in which the tumors develop is under the control of the pituitary gland, it was of interest to investigate the possibility of a pre-carcinogenic change in the protein-labeling rate of the pituitary gland.

The data obtained on methionine incorporation by proteins of pituitary tissue are presented in Table 5. It is obvious that the over-all activity of the pituitary tissue from the tumor-susceptible females is identical to that obtained from the foster-nursed female controls. On the other hand, the male glands are about 25 per cent less active than those of the females, and this difference is found to be statistically significant ($p = 0.05$). This is in contrast to the results obtained with albino rats, where no sex difference in protein-labeling by pituitary was observed (13).

The results have also been calculated in terms of total uptake per kg. of animal. It is apparent that the sex difference becomes even greater when expressed in this fashion, the male showing about 40 per cent less total activity than that of the C3H females. In addition, the foster-nursed animals show a slightly greater over-all incorporation of the label than do the tumor-susceptible females.

This is explained by the data on the size of the pituitary in this strain, which is presented in Table 6. It is apparent that the male glands are smaller

TABLE 4

EFFECT OF ESTRONE IN *in Vivo* ON MALE SKIN*

Treatment	Methionine incorporation μM/kg/hr
None (14)	81 ± 4
Mazola injections (4)†	92 ± 5
Estrone injections (8)†	86 ± 7

* See footnotes to Table 1.

† Ten to 50 injections were given, as described in text.

TABLE 5

INCORPORATION OF METHIONINE BY PITUITARY TISSUE

Group*	Methionine uptake μM/kg wet wt/2 hr	Methionine uptake μM/kg body wt/2 hr
C3H male (6)	265 ± 15†	1.46 ± 0.10
C3H female (6)	328 ± 32	2.38 ± 0.22
Foster-nursed females (6)	326 ± 15	2.62 ± 0.09

* The number of determinations is indicated in parentheses. Each determination was done on 4–6 pooled glands.

† Plus or minus the standard error of the mean.

than those of the female—this is in accord with data collected from other species (13). The striking difference in average weight of the pituitary between the tumor-susceptible females and the foster-nursed females was completely unexpected. It was found, however, that the average body weights of the two groups were also significantly different. As an example of this discrepancy, we found, in a group of animals 5–6 months of age, 25 females with an average weight of 23 gm. and 24 foster-nursed females with an average weight of 28 gm. The animals had been raised under slightly different conditions of crowding and storage before receipt by our colony at 4–5 weeks of age.[1] However, the two groups differed in average weight by only 1 gram upon receipt and had identical treatment after arrival in our colony; they appeared to be in good health throughout the course of this work. No conclusion can be drawn

from this observation, since the conditions under which it was made were not specifically controlled for growth studies.

In view of the above, the data on pituitary weight are expressed in terms of μg/unit body weight. It is apparent that a significant difference in pituitary size between the two groups of females exists even when the data are calculated on this basis ($p \ll 0.001$). It appears that, in this group of 94 animals, the C3H females have, on the average, 13 per cent less pituitary tissue per gm. body weight than genetically identical animals which differ only by lacking the Bittner milk factor. This would seem to indicate a pre-cancerous change in these animals which is related specifically to the milk factor.

TABLE 6

PITUITARY SIZE IN C3H MICE*

ANIMAL GROUP	PITUITARY WEIGHT mg.	PITUITARY MICROGRAM/GRAM ANIMAL μg/gm animal
C3H male (44)	1.53±0.04	54.3±1.4
C3H female (55)	1.68±0.04	72.2±1.4
Foster-nursed female (39)	2.26±0.04	82.5±1.3

* See footnotes to Table 1.

SUMMARY

1. A study has been made of the incorporation of S^{35}-labeled methionine *in vitro* by the tissues of C3H mice. The observed relative rates are mammary tumor > pituitary > mammary tissue > skin > liver.

2. No difference in the rate of incorporation was observed in skin preparations from C3H female mice, C3H male mice, or foster-nursed C3H females.

3. No effect of estrone added *in vitro*, or of repeated injections of estrone prior to sacrifice, could be observed on the process in skin or mammary tissue.

4. Estrone and testosterone added *in vitro* had no effect on protein-labeling by tumor slices.

5. The incorporation of labeled methionine by pituitary tissue was studied. Glands taken from males were found to be significantly less active than those from females. The presence or absence of the milk factor in females had no effect on the incorporation of the label into protein.

6. The pituitaries of the male animals are smaller than those of the other two groups. In addition, the C3H females have a gland which is 13 per cent smaller per gram body weight than that of foster-nursed females.

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3-Methylcholanthrene as an Inhibitor of Hepatic Cancer Induced by 3′-Methyl-4-dimethylaminoazobenzene in the Diet of the Rat: A Determination of the Time Relationships*†

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One of the more interesting recent developments in experimental cancer research is the observation that one carcinogenic agent may inhibit another (11, 23, 25). A well known agent for the production of experimental hepatic cancer is 3′-methyl-4-dimethylaminoazobenzene (*m′*-Me-DAB).[1] Methylcholanthrene (MCA) is a powerful carcinogen capable of producing sarcomas when injected into the skin of rats and epidermoid carcinomas when painted on cutaneous surfaces. Richardson and co-workers (18, 19) found that MCA given simultaneously with *m′*-Me-DAB in the diet of the rat would inhibit the induction of hepatic tumors by the latter.

The purpose of the present experiment was to determine the effectiveness of the delayed administration of MCA, as is manifested by (*a*) the reversibility of the changes initiated by *m′*-Me-DAB, (*b*) the correlation between the time of the appearance of the morphological liver changes with *m′*-Me-DAB and the time of initiation of MCA feeding, and (*c*) the effect of MCA upon those sequential hepatic changes ("pre-cancerous") occurring prior to the carcinomas.

MATERIALS AND METHODS

Diets.—The basic synthetic diet was that formulated by Griffin *et al.* (7). To this diet *m′*-Me-DAB was added at a level of 0.06 per cent. MCA was mixed with the *m′*-Me-DAB diet in 0.0067 per cent concentration.

Animals.—Two hundred and sixty albino rats of the Sprague-Dawley strain, which had been maintained on a commercial laboratory chow,[2] were divided into groups of ten to fifteen animals and placed on the basal synthetic diet to which 0.06 per cent *m′*-Me-DAB had been added. Males and females were used in approximately equal numbers, and the initial weights of the animals ranged from 200 to 300 gm. At intervals from 3 weeks up to 18 weeks after the initiation of the feeding of *m′*-Me-DAB, MCA was added to the diet. The animals were then maintained throughout the remainder of the experiment on the diet containing the two carcinogens. Animals from each group of animals receiving *m′*-Me-DAB plus MCA were sacrificed at 3-week intervals. Two control groups were established, one for the basic diet and another for the *m′*-Me-DAB diet. The animals on *m′*-Me-DAB were killed at weekly intervals. The animals on the basic diet were utilized as controls for spontaneous hepatic changes and for adequacy of nutrition. They were sacrificed after 13–37 weeks on the experiment. All groups were studied concurrently as one large experiment.

Technic of autopsy.—The animals were killed with ether. Two and one-half ml. of blood were withdrawn from the inferior vena cava and placed in a test tube containing a measured amount of dried ammonium and potassium oxalate. The hematocrit, hemoglobin, and plasma proteins were determined by the copper sulfate method (20).

In the preparation of liver smears two small sections were immediately taken from areas of the liver with gross changes. If no changes could be detected, an area from the right lobe and an area from the left lobe were chosen. The liver was crushed and smeared on a glass slide with a spatula and immediately placed in a Coplin jar containing Vandegrift's fixative (24). They were stained with eosin, orange G, and hematoxylin.

The pituitary, thyroid, parathyroid, trachea, esophagus, lungs, stomach, pancreas, liver, spleen, kidneys, adrenals, ovaries, tubes, uterus, and testes were studied grossly and microscopically. Fat stains were made on the adrenal glands. The weights of the animals and of the adrenals, pituitaries, and livers were recorded. The tissues were fixed immediately with Vandegrift's fixative, and they were stained with eosin, orange G, and hematoxylin.

RESULTS

Description of pathologic changes within the liver.—To indicate the criteria used in this paper for the different pathologic changes in the liver, a brief description and illustration of each is presented.

The earliest change consists of cirrhosis. In its mild or early stage the only gross alteration is a slight pallor. Microscopically one sees a proliferation of fibroblasts and a deposition of collagen, beginning in the portal areas and extending out through the interlobular spaces (Fig. 1). There is

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† With another numbering system 3-methylcholanthrene is referred to as 20-methylcholanthrene.

[1] We are indebted to Dr. A. Clark Griffin of the Department of Chemistry, Stanford University, for the supply of the dye used in these experiments.

[2] Purina Laboratory Chow, Ralston Purina Company, St. Louis, Missouri.

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usually no distortion of the lobule until the cirrhosis has advanced to a moderate degree, at which time the cirrhosis is readily detectable grossly.

Bile duct proliferation as a morphologic change is to be distinguished from cirrhosis, since it does not usually occur with the mild stages of the latter (Fig. 2). Bile duct proliferation accompanies the later stages of cirrhosis. Groups of bile ducts may show marked dilatation; these have been termed "bile duct cysts" (Fig. 2).

Usually, while the bile duct proliferation is becoming more severe, benign hepatomas appear. The term "benign hepatoma" has been previously applied to nodular hyperplastic changes of the parenchymal cells similar to that seen in human cirrhosis. In our experimental animals these changes ranged from focal alterations within a lobule to larger, well circumscribed lesions without remaining lobular pattern (Figs. 3 and 4). Large to small lesions occur concomitantly. Fibrous tissue is seen only at their periphery. The cells of such "benign hepatomas" are characterized by their large size, their pale, vacuolated cytoplasm, and their slightly enlarged nuclei with prominent nucleoli. These cells appear to be identical to those which have been termed "drug-effect cells" by Smith and Richardson (17).[3]

Grossly a large benign hepatoma cannot be accurately distinguished from a carcinoma. When early carcinoma is identifiable, however, it is a white, hard, and solitary nodule. The advanced carcinomas are identifiable by the greatly enlarged liver, marked distortion of the lobes, and the numerous larger nodules, some of which are necrotic. The carcinomas never occur without accompanying cirrhosis, which usually is severe, but occasionally may be mild. Bile duct proliferation is also present. Hepatic carcinomas are usually accompanied by benign hepatomas. The carcinomas may be of two general types, those arising from bile ducts and those arising from the parenchymal or cord cells. The classification and the description of these malignant hepatic tumors have been presented in detail by other investigators (4, 9, 19).

Effects of the basic diet.—The animals on the basic diet were followed until the 36th week. The liver tissue could not be distinguished from that of normal laboratory animals (Fig. 5). No significant differences were noted in the fat content or its distribution in the adrenal glands, the organ weights, hemoglobin, hematocrit, or plasma proteins. The only tissue change was the chronic lung disease seen in the majority of the laboratory animals. This, however, was of no greater incidence than in the colony controls.

Effect of the m′-Me-DAB diet.—There was an initial weight loss during the first 2 weeks, but after this time the animals began to gain weight. Between the 3d and the 9th weeks a considerable number of animals rapidly became debilitated and died. Liver changes were minimal during this time, and death was usually attributable to severe pulmonary disease, but hepatic insufficiency could not be eliminated as a factor. In those animals sacrificed on schedule, milder pulmonary disease was present. In these latter animals no significant differences were noted in the organ weights, blood work, or adrenal studies.

At the 6th week, the first interval of observation after initiation of feeding of *m′*-Me-DAB, all animals showed cirrhosis of a mild to severe degree. Bile duct proliferation was seen in the majority of animals, and benign hepatomas were common. An unexpected finding was a carcinoma in one animal sacrificed at the 6th week.

After the 9th week all the animals but one had benign hepatomas. Carcinomas were found sporadically up to the 18th week. At this time, and thereafter, all animals had hepatic cancers (Figs. 6 and 7) except two, one sacrificed at the 27th week and one sacrificed at the 39th week (Charts 1 and 2).

The carcinomas most commonly metastasized to the splenic lymph nodes and to the lungs. Other sites of metastases were the pancreas, spleen, and omentum (Fig. 8).

Effects of delayed addition of MCA to animals on m′-Me-DAB.—General remarks: Animals which received MCA before the 10th week had a much lower mortality rate than did those on the *m′*-Me-DAB diet. As was observed in animals receiving only *m′*-Me-DAB, there was a slight loss in weight after the addition of MCA, but after 2–3 weeks the animals began to gain weight.

In the adrenal cortex many changes in the numbers of vacuoles, size of cells, compactness of the zona glomerulosa, and vascularity of the gland could be noted. The fat content of the adrenal cortex was studied with Oil Red O and Oil Blue N as suggested by Wilson (27). There was no correlation between any histologic change and the duration of administration of either carcinogen. The adrenal weights and adrenal to body weight ratios showed no consistent trend.

Changes in the individual liver cells in the smear preparations could not be relied upon for a diagnosis of the type of diet the animal had received. The blood work showed no consistent changes which could be correlated to the administration of

[3] Marthe Smith and H. L. Richardson, personal communication.

the carcinogen or the inhibitor. The gross appearance of the livers in these groups was remarkably better than of those from rats on the *m'*-Me-DAB diet.

MCA given after 3–5 weeks on *m'*-Me-DAB: No carcinomas were found (Charts 1 and 2). Benign hepatomas occurred, but their appearance was delayed. Cirrhosis occurred in all animals, but it was of milder degree than that observed in the *m'*-Me-DAB controls.

MCA given after 6–10 weeks on *m'*-Me-DAB: Carcinomas were found in groups 6, 8, 9, and 10, but the appearance was delayed, and the incidence was lower than in those animals receiving the *m'*-Me-DAB diet without MCA (Charts 1 and 2). In Group 7 no carcinomas occurred. Benign hepatomas were found in all of these groups, but they occurred at a later time in the experiment than in animals receiving the diet containing *m'*-Me-DAB alone. Cirrhosis appeared in all groups, but the severity was less than that found in the *m'*-Me-DAB animals.

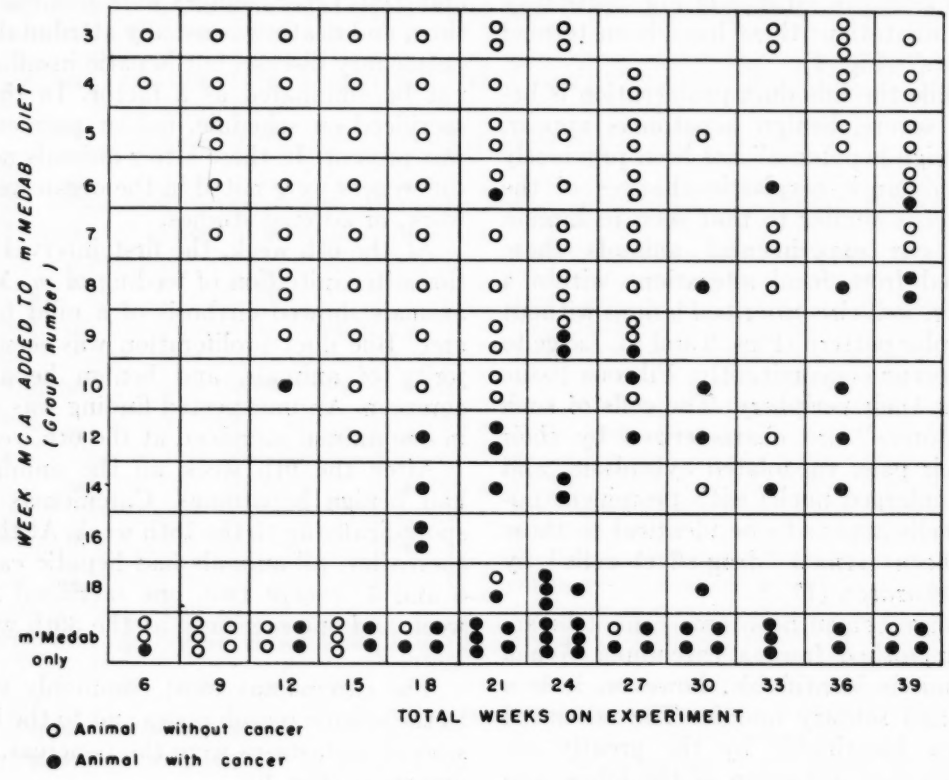

CHART 1.—Cancer incidence in rats on the various diets

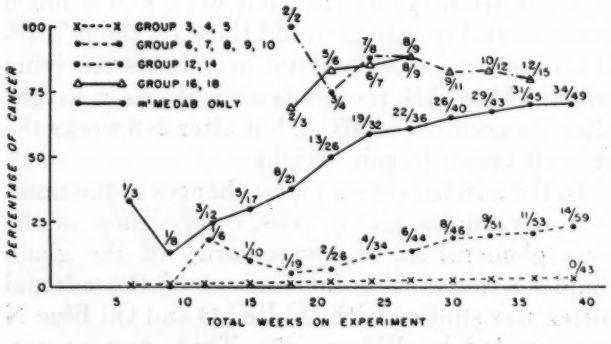

CHART 2.—Cumulative percentage of animals with cancer

MCA given after 12 weeks on *m'*-Me-DAB: There were no significant differences in the number of carcinomas or benign hepatomas or in the time of their appearance in these animals as compared to those receiving *m'*-Me-DAB alone (Fig. 9, Charts 1 and 2).

DISCUSSION

Besides the production of hepatic carcinoma by the *m'*-Me-DAB diet, there are other morphologic changes in the liver which have some sequential relationship. Thus, cirrhosis appears first; this is followed by bile duct proliferation, then benign hepatomas, and finally carcinomas. In general, the earlier changes increase in severity while the later changes are making their appearance.

The present experiment, while dealing primarily with the inhibition by MCA of the induction of carcinoma by *m'*-Me-DAB, also revealed that the

earlier morphologic changes in the liver were inhibited. Richardson, Stier, and Borsos-Nachtnebel (19) found that simultaneous administration of MCA and *m'*-Me-DAB inhibited even the earliest of these sequential changes in the *liver*, i.e., cirrhosis. In the present study cirrhosis always occurred when MCA administration was delayed over 3 weeks. However, the cirrhosis was of a milder form. The bile duct proliferation tended to be less marked, benign hepatomas were delayed in their appearance, and carcinomas could be prevented by the addition of MCA up to the 5th week and definitely inhibited by addition up to the 10th week.

The time of administration of MCA may be roughly correlated with the time at which the various changes might be expected from *m'*-Me-DAB alone. Inhibition of carcinoma became progressively less effective the later the MCA was added to the *m'*-Me-DAB diet. The inhibition of carcinoma, however, was more effective than the inhibition of cirrhosis, bile duct proliferation, and benign hepatomas. Since the cirrhosis was already established by the 6th week on the *m'*-Me-DAB diet, one would not expect to obtain inhibition of cirrhosis by giving MCA later than the 6th week. This same generalization extends to bile duct proliferation, benign hepatomas, and carcinomas. From this correlation it is evident that MCA will not reverse a process that already has been established but will delay the progression of this stage.

Knowledge of the mechanism through which MCA alters the hepatotoxic effects of *m'*-Me-DAB may afford some insight concerning the pathogenesis of carcinomas. The ultimate mechanism of action is unknown, but it is probably a combination of various endocrinological (8, 14, 19), chemical (1, 2, 5, 10, 13, 15, 21, 22, 26), and morphological (3, 4, 6, 16) aspects. In an abstract Miller *et al.* (12) presented data which suggest that the hydrocarbons might inhibit azo dye carcinogenesis by maintaining the ability of the rat liver to metabolize the dyes and thereby reduce the effective dose of the dye in the liver. Too little is known of these processes to warrant any detailed discussion in this paper.

SUMMARY

A basal diet containing 0.06 per cent 3'-methyl-4-dimethylaminoazobenzene was fed to albino rats of the Sprague-Dawley strain, and at intervals of 3 to 18 weeks 3-methylcholanthrene was added to the diet in a concentration of 0.0067 per cent. The animals were sacrificed at regular intervals to detect the sequential changes occurring in the livers.

It was found that 3-methylcholanthrene would prevent carcinoma of the liver if it were added to the diet before the 6th week, and that there was partial inhibition if the 3-methylcholanthrene were added before the 10th week of administration of 3'-methyl-4-dimethylaminoazobenzene. After the 10th week no inhibition resulted.

ACKNOWLEDGMENTS

We are indebted to Dr. Howard L. Richardson for his help and advice in the initiation of this experiment, and to Dr. Carl Hopkins for his statistical analysis and suggestions for presentation of the experimental data.

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Fig. 1.—Mild cirrhosis in a rat on *m′*-Me-DAB 33 weeks and MCA 26 weeks. (Compare with Fig. 9 of a rat on *m′*-Me-DAB 34 weeks.) ×100.

Fig. 2.—Bile duct proliferation and cysts in a rat on *m′*-Me-DAB 6 weeks and MCA 2 weeks. ×100.

Fig. 3.—Moderate cirrhosis and a small, benign hepatoma in a rat on *m′*-Me-DAB 11 weeks and MCA 4 weeks. ×100.

Fig. 4.—Two benign hepatomas in a rat on *m′*-Me-DAB 18 weeks and MCA 9 weeks. ×100.

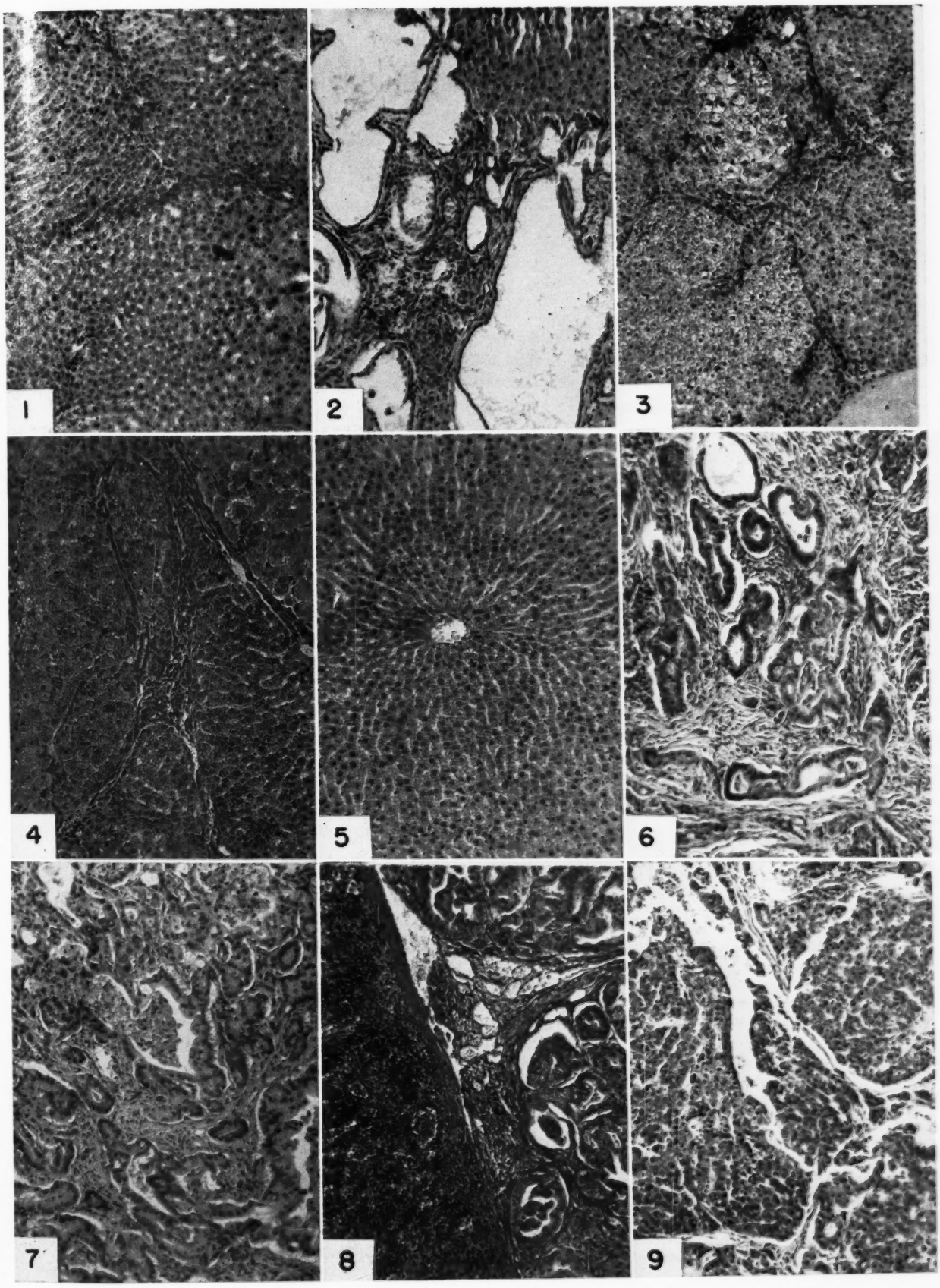
Fig. 5.—Normal liver of a rat on the basic diet 17 weeks. ×100.

Fig. 6.—Adenocarcinoma in a rat on *m′*-Me-DAB 34 weeks. ×100.

Fig. 7.—Adenocarcinoma in a rat on *m′*-Me-DAB 30 weeks. ×100.

Fig. 8.—Metastatic extension of a hepatic carcinoma to the spleen of an animal on *m′*-Me-DAB 23 weeks. ×100.

Fig. 9.—Hepatic carcinoma in a rat fed *m′*-Me-DAB 14 weeks before MCA was added (*m′*-Me-DAB 24 weeks and MCA 10 weeks). ×100.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Aerobic Glycolysis in Homogenates of Normal and Tumor Tissues*

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Previous investigations from this laboratory have revealed that the over-all oxidative *capacity* of tumor tissues, in general, falls in the range of the less active normal tissues and at a level much lower than normal liver, kidney, heart, or brain (12), while the enzymes of the glycolytic system occur in quantities of the same order of magnitude in all these tissues (5, 6). Since the conclusions regarding oxidative capacity have been questioned (19), further studies with systems not previously employed are here reported.

In earlier studies on the malic dehydrogenase system (10, 16), information was obtained concerning the DPN[1]-cytochrome c reductase content of various tissues. This was done by adding a crude preparation of malic dehydrogenase that contained no cytochrome reductase to a homogenate, with the result that the rate-limiting reaction was the reduction of cytochrome c and that the measurement of oxygen uptake could be taken as an assay of the DPN-cytochrome c reductase when DPN and cytochrome c were added in excess. The data showed that in liver homogenates the cytochrome c reductase was present in excess, and it was concluded that, in the absence of added malic dehydrogenase, the rate of oxygen uptake was a measure of the latter enzyme in the homogenate. However, in primary rat hepatomas there was such a narrow margin between the two enzymes that it could not be assumed that either enzyme was limiting in the unsupplemented homogenate (10, p. 317). More recent work by Weinhouse *et al.* (21, 22) has indicated that the malic dehydrogenase component is not low in tumor tissue, and this observation suggests that the cytochrome reductase was limiting even in the measurements carried out without a malic dehydrogenase supplement. These studies yielded Q_{O_2} values of 103.5 and 15.1, respectively, for liver and hepatoma (10).

Reaction mixtures for the study of glycolysis in tumor homogenates have been perfected by LePage (5) and have shown that the glycolytic enzymes are present in tumor homogenates at a level that should deliver sufficient reduced DPN to saturate the DPN-cytochrome c reductase system in tumor tissue at the levels reported earlier (10). Thus, the rate of oxygen uptake in a properly supplemented tumor homogenate carrying out aerobic glycolysis might be used as a measure of the DPN-cytochrome c reductase or of the over-all DPN-oxidizing capacity of the tissue. The present report deals with the properties and limitations of such a system.

MATERIALS AND METHODS

Normal tissues were taken from 200–250-gm. male albino rats.[2] Flexner-Jobling carcinomas and Walker 256 carcinosarcomas were taken 10 days after subcutaneous transplantation into 120–150-gm. female rats of the same strain, while Jensen sarcomas were excised 6 days after transplantation. Mouse Ehrlich ascites tumors were harvested 5 days after transplantation. The Novikoff hepatoma was recently obtained from Dr. A. B. Novikoff of the University of Vermont. It was transplanted intraperitoneally and grew rapidly to a weight of 10–15 gm. of non-necrotic tissue in 7–9 days. It was originally obtained by transplanting a primary liver tumor that arose after feeding 4-dimethylaminoazobenzene.

Normal and tumor tissues were rapidly excised from decapitated rats and placed in small beakers containing isotonic sucrose (0.25 M) and standing in cracked ice. The tissues were homogenized with a suitable volume of cold isotonic sucrose in a pestle-homogenizer. The ice-cold homogenates were pipetted into Warburg vessels standing in cracked ice and already containing all other constituents of the system. The vessels were then attached to the manometers without delay with air as the gas phase. At the end of the incubation period, 0.1 ml. of 50 per cent perchloric acid was pipetted into each flask to precipitate the proteins. After centrifuging, the supernatant fluid was analyzed for inorganic phosphate by the method of Fiske and Subbarow and for lactic acid by the method of Barker and Summerson, both as described by LePage (18).

HDP was obtained as the barium salt from Schwartz Laboratories, Inc., New York, while adenosine ATP was obtained as the barium salt from the Pabst Laboratories, Milwaukee. In each case, the barium was precipitated with sulfuric acid and the free acids were neutralized with potassium bicarbonate and sodium hydroxide, respectively. The DPN used in these experiments was prepared by the method of LePage (18) and assayed as 85 per cent DPN.

* A preliminary report has been given (14).

[1] Abbreviations used: DPN = diphosphopyridine nucleotide, HDP = fructose 1:6-diphosphate, BCB = brilliant cresyl blue, ATP = adenosine triphosphate.

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[2] Supplied by the Holtzman-Rolfsmeyer Company of Madison.

RESULTS

Reaction Mixture for the Aerobic Glycolysis of HDP

In order for glycolysis to proceed from HDP to lactic acid, oxidized DPN must be available to mediate the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. The two main pathways of hydrogen transport for the re-oxidation of reduced DPN are presumably (*a*) by DPN-cytochrome c reductase and the cytochrome system to oxygen and (*b*) by coupling with the enzymatic reduction of pyruvate to lactate. LePage has shown (5) that the glycolysis of HDP under anaerobic conditions and in the absence of fluoride is stimulated only 27 per cent by the addition of pyruvate; this proves that sufficient oxidized DPN is present, even under anaerobic conditions, to initiate the glycolysis of HDP. Once pyruvic acid has been formed, DPNH can be reoxidized, and glycolysis can continue unhindered.

TABLE 1

Effect of Substrate (HDP) and Potassium Bicarbonate on Oxygen Uptake in Heart Homogenates

Each compound was studied at varying concentrations, with all other components held constant at the values given in Table 3. The concentrations selected for assay are indicated by italics. A 10 per cent homogenate of rat heart in isotonic saline was used as source of enzyme and Q_{O_2} values are based on oxygen uptake readings during the first 10 minutes of the experiment.

HDP		KHCO	
Molarity	Q_{O_2}	Molarity	Q_{O_2}
0	14	0	51
0.0067	39	0.001	61
0.013	*62*	*0.003*	*65*
0.020	67	0.005	59
0.033	69		
0.053	63		

To demonstrate the full oxidative capacity of the DPN-cytochrome c reductase system at the 3-phosphoglyceraldehyde step, a complete break of the coupling with the pyruvate system would be necessary. This can best be achieved in the absence of pyruvate. While complete suppression of pyruvate formation is not possible, the quantity of pyruvate is a minimum at the start of the reaction period, and a fluoride block at the phosphoglyceric acid level inhibits pyruvate formation. Under these conditions, the oxygen uptake of the glycolytic system should be a measure of DPN-cytochrome c reductase, since the glycolytic enzymes have been demonstrated to be very active in homogenates of normal and neoplastic tissues (6).

A system for the aerobic glycolysis of HDP has already been described by Potter (11), while a system for the anaerobic glycolysis of HDP has been described by LePage (5, 6). In the present series of experiments an attempt has been made to adjust the concentrations of the various reactants used previously to secure optimum conditions for oxygen uptake. Unless otherwise specified, a homogenate of rat heart in isotonic potassium chloride was used as source of enzymes in the following experiments.

Substrate.—Table 1 shows experiments on which the choice of substrate concentrations used in the assay medium were based. The minimum substrate concentration required to give close to maximal oxygen uptake was chosen.

Bicarbonate.—Table 1 shows the effect of potassium bicarbonate, which was added to neutralize lactic acid produced in the process of glycolysis. Carbon dioxide liberated from the bicarbonate was absorbed by alkali placed for that purpose in the center-well of the Warburg flasks. This procedure has been discussed elsewhere (11). Table 1 shows that a fivefold variation in bicarbonate did not greatly affect the optimum result.

Pardee center-well mixture.—The effect of the Pardee center-well mixture (8) was tested in the system. This mixture is capable of maintaining a constant pressure of carbon dioxide within the flask, when used in conjunction with an appropriate level of bicarbonate in the medium. However, experiments with a carbon dioxide pressure of ½ per cent, 1 per cent, 3 per cent, and 5 per cent did not constitute an improvement over the usual center-well addition of alkali.

pH of buffer.—Addition of phosphate buffer to provide substrate for phosphorylation reactions was held to a minimum in order to facilitate accurate measurements of changes in inorganic phosphate during the course of glycolysis. No appreciable effect was obtained by varying the pH of the phosphate addition from pH 7.4 to pH 8.2.

ATP.—ATP was added as a source of high energy phosphate bonds at a level previously used (5). Doubling this level of ATP had no effect on oxygen uptake.

Fluoride.—Fluoride inhibits ATP-ase activity (11) and also inhibits glycolysis at the phosphoglyceric acid stage. Fluoride was added at a previously determined level (11), which produced considerable stimulation of oxygen uptake (9), as experiments reported in a later section show. Trebling this level of fluoride caused inhibition of oxygen uptake.

Cytochrome c.—Cytochrome c has been shown to stimulate oxygen uptake in this system and was added at a level close to that previously recommended (11); doubling this level had no effect on

oxygen uptake. Further experiments on the effect of cytochrome c are reported in a later section.

Homogenizing media.—The effect of different homogenizing media on oxygen uptake was studied with liver from a rat starved for 24 hours as source of enzyme. The Q_{O_2} values obtained with 10 per cent homogenates in water, isotonic potassium chloride, and isotonic sucrose were, respectively, 20.2, 20.0, and 20.5, showing essentially no difference between the different media.

Tonicity.—The effect of tonicity on oxygen uptake was studied with the same liver preparation. Table 2 shows that a slight inhibition was obtained

TABLE 2

EFFECT OF TONICITY ON OXYGEN UPTAKE IN LIVER HOMOGENATES

Sucrose additions were used to increase tonicity. A 10 per cent homogenate of rat liver in isotonic sucrose was used as source of enzyme, and Q_{O_2} values are based on oxygen uptake readings during 60 minutes. See text.

Tonicity	0.63	0.80	1.0	1.20	1.50
Q_{O_2}	21.5	21.3	20.9	20.6	19.1

with increasing tonicity. No addition was therefore made to raise the level of tonicity in the medium above the value of 0.63 that was due to its requisite constituents. In this table, isotonic sucrose (0.25 M) was given a value of 1.0. The nonsucrose additions were calculated to osmotic equivalents on the basis of their ionic strengths assuming 100 per cent ionization, and the final tonicity was adjusted with sucrose.

Creatine.—Although creatine has been shown to affect phosphate esterification in a similar system (11), no appreciable effect on oxygen uptake was obtained by the addition of 30 mg creatine/flask with either heart or kidney homogenates. Creatine was therefore omitted from the reaction mixture.

Arsenate.—Arsenate at a final concentration of 0.0033 M slightly stimulated oxygen uptake, while at 0.01 M a small initial inhibition was obtained. However, the initial level of oxygen uptake declined less rapidly than in control flasks. These effects did not warrant the addition of arsenate to the reaction mixture.

DPN.—Chart 1 shows the effect of increasing DPN concentrations on the Q_{O_2} (μl O_2 uptake/mg dry weight of tissue/hour) of one normal and three neoplastic tissues. On a 10-minute basis, near-maximal oxygen uptake was obtained at a level of 0.1 ml. of 0.4 per cent solution of DPN, or a final concentration of 2.2×10^{-4} M. On a 60-minute basis, this level of DPN was still satisfactory except in the case of Jensen sarcoma, where a higher level was more appropriate. In view of the depression of oxygen uptake readings with time (see below) which is probably due to the already mentioned production of pyruvate during the course of glycolysis, Q_{O_2} values based on the first 10 minutes were thought to be the relevant values; to obtain these, a DPN concentration of 2.2×10^{-4} M was adequate (see footnote to Table 3).

Nicotinamide.—Chart 2 shows the effect of nicotinamide, which acts as a noncompetitive inhibitor of DPN-ase (24) to preserve DPN that is otherwise rapidly broken down in homogenates (7). On a 10-minute basis, 0.1 ml. of a 1.2 M solution of nicotinamide is optimal for all tissues tested, while on a 60-minute basis 0.1–0.2 ml. is optimal. The choice for the assay system was the lower level; it corresponds to a final concentration of 0.04 M.

Tissue concentration.—Chart 3,*a* shows that the oxygen uptake declines with time. With all tissues tested, this decline was smaller at low than at high tissue levels. Chart 3,*b* shows that a direct proportionality exists between oxygen uptake and tissue concentration.

The composition of the medium for the aerobic glycolysis of HDP is given in Table 3.

TABLE 3

COMPOSITION OF THE AEROBIC GLYCOLYSIS MEDIUM

Addition	Final Molarity
Hexosediphosphate*	0.010
Adenosine triphoshate†	0.0010
Diphosphopyridine nucleotide‡	0.00022
Nicotinamide	0.040
Cytochrome c	0.00002
Magnesium chloride	0.0033
Potassium fluoride	0.010
Potassium bicarbonate (fresh)	0.0033
Potassium phosphate, pH 7.4	0.0033
Tissue homogenate in 0.25 M sucrose	
Water to make a final volume of 3.0 ml.	
Potassium hydroxide (2.0 M): 0.2 ml. in center well	
Gas mixture: air	

* Potassium salt.

† Sodium salt.

‡ This concentration was chosen after initial experiments with heart homogenate as the source of enzyme. Later tests with several tissues, shown in Chart 1, indicated that for one tissue, Jensen sarcoma, a higher DPN concentration would be more appropriate to obtain near-maximal oxygen uptake over an extended period such as an hour. On this account, a DPN concentration of 6×10^{-4} M is recommended for future work.

TISSUE DIFFERENCES IN OXYGEN UPTAKE

Table 4 shows the results obtained when the reaction mixture given in Table 3 was used to compare the oxygen uptake of different tissues. Column 1 of Table 4 shows a grading of DPN-cytochrome c reductase capacity from higher values for normal tissue to lower values for neoplastic tissues.

Effects of cytochrome c and BCB.—Column 3 of

Table 4 shows that omission of cytochrome c depresses the oxygen uptake of all tissues except the Ehrlich ascites tumor.

BCB acts in this and related systems as a direct link between diaphorase and oxygen (17), thereby providing a pathway for the oxidation of DPNH that bypasses DPN-cytochrome c reductase. Column 4 of Table 4 shows that, for the four normal tissues tested, BCB had no effect on oxygen uptake. In contrast, the oxygen uptakes of the four neoplastic tissues were raised to a mean value of 190 per cent of control. The result obtained with Jensen sarcoma stresses the desirability of working with several different types of neoplastic tissues, before drawing conclusions of a generalized nature.

The above results show that BCB strongly stimulates the oxygen uptake of three out of the four tumor tissues studied, while producing little or no effect with normal tissues. These results represent further evidence that the capacity of the

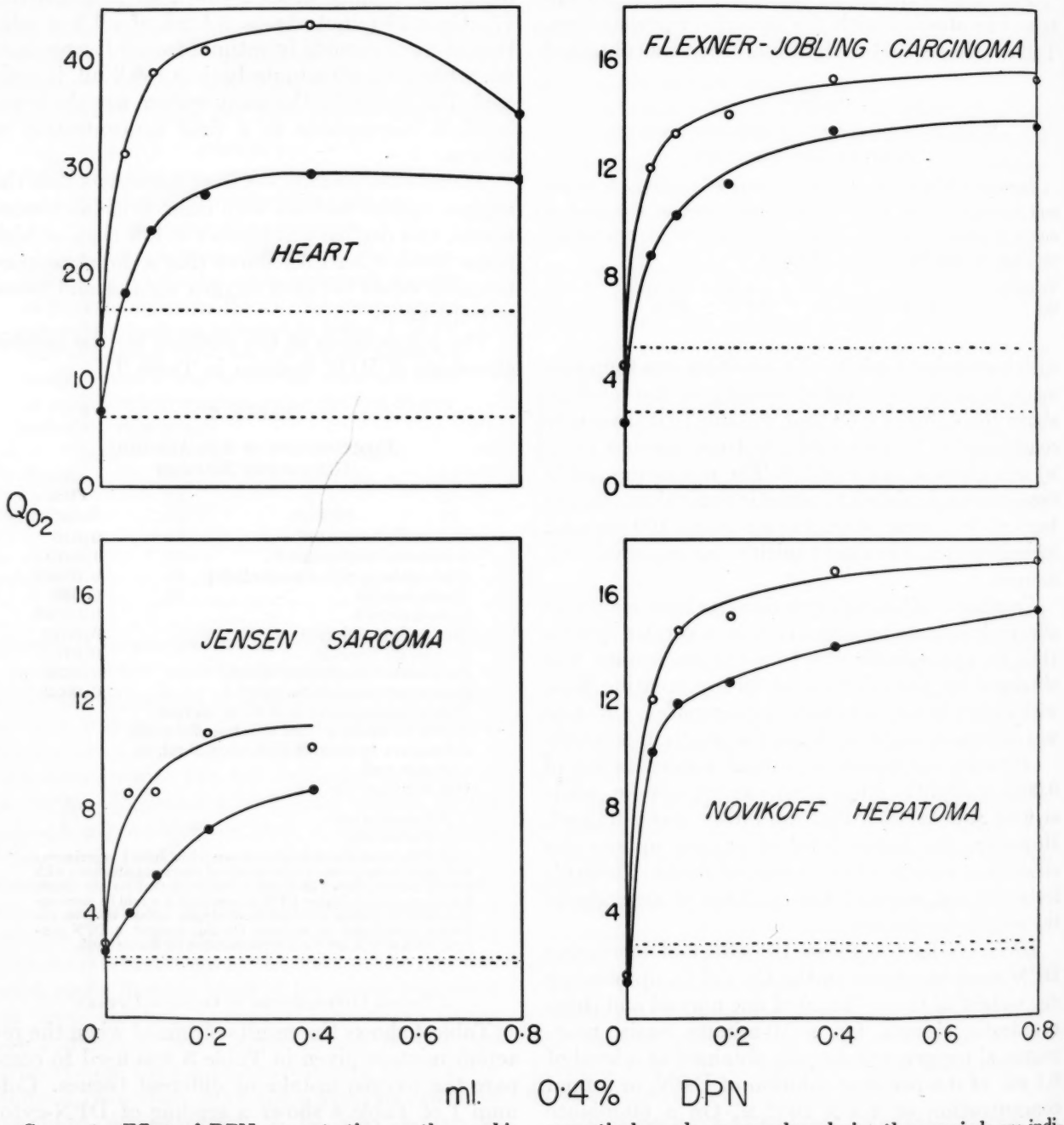

CHART 1.—Effect of DPN concentration on the aerobic glycolysis of HDP by homogenates of normal and tumor tissues. Oxygen uptakes during the first 10 minutes and during the first hour are represented by open and closed circles, respectively; endogenous values during these periods are indicated by the corresponding dotted lines. The highest level of DPN (0.8 ml. of a 0.4 per cent solution) corresponds to a final concentration of 1.8×10^{-3} M.

DPN-cytochrome c reductase is limited in these tumors as compared to normal tissues.

Column 5 shows the effect of adding BCB in the absence of cytochrome c. It is seen that only two tissues, namely, Flexner-Jobling carcinoma and Walker 256 carcino-sarcoma, have their oxygen uptake stimulated by both cytochrome c (compare columns 1 and 3) and BCB (compare columns 5 and 3); and for both these tissues the presence of both cytochrome c and BCB is necessary for maximal oxygen uptake (compare column 4 with 1 or 5). This suggests that addition of BCB presents an alternative pathway for hydrogen transport but leaves intact, at least to some degree, the original pathway via cytochrome c.

Effect of Inhibitors and Substrates on Lactic Acid Production and Phosphate Release

Tables 5 and 6 represent a survey of glycolytic reactions in a number of normal and neoplastic

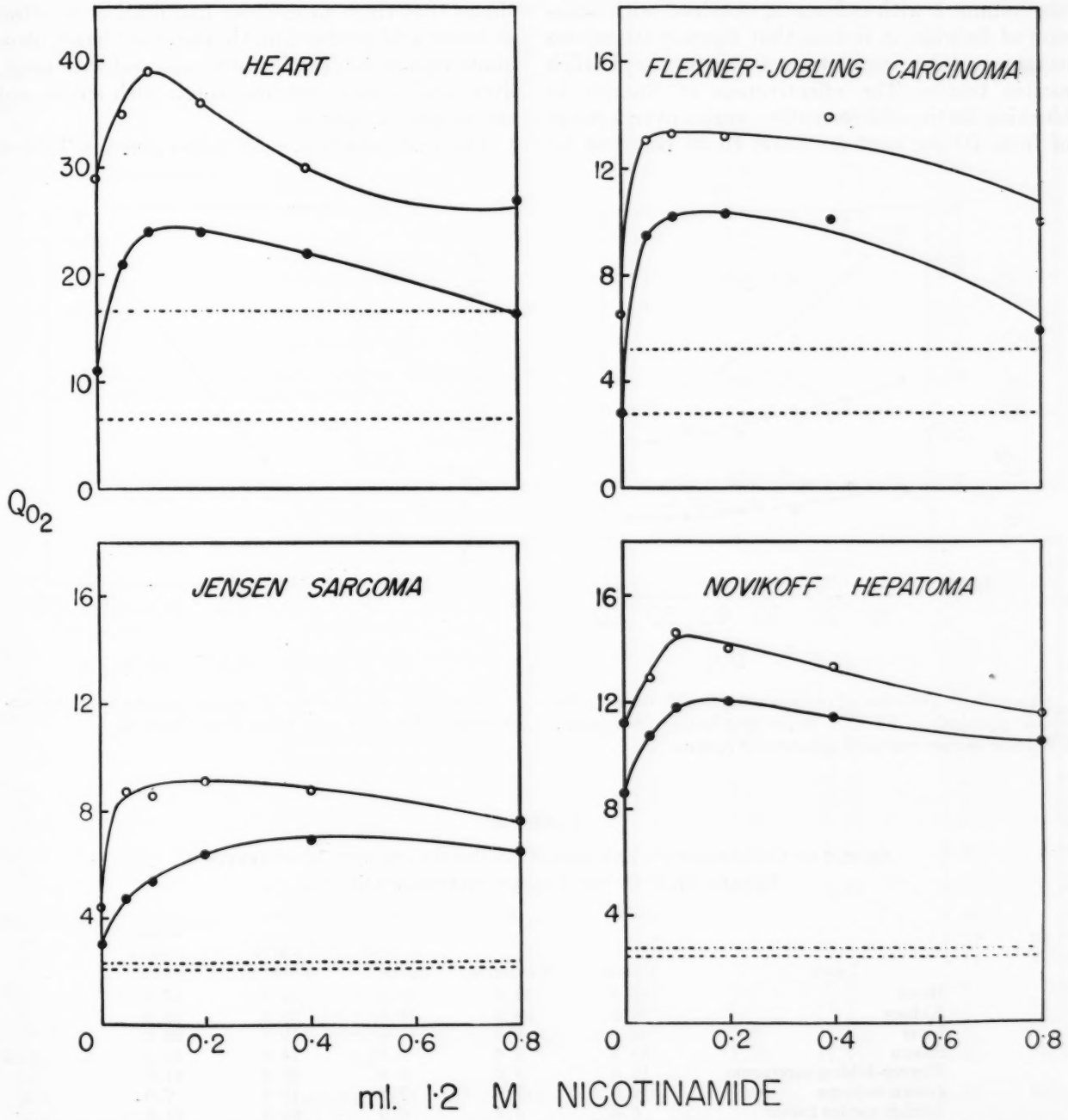

CHART 2.—Effect of nicotinamide concentration on the aerobic glycolysis of HDP by homogenates of normal and tumor tissues. Oxygen uptakes during the first 10 minutes and during the first hour are represented by open and closed circles, respectively; endogenous values during these periods are indicated by the corresponding dotted lines. The highest level of nicotinamide (0.8 ml. of a 1.2 M solution) corresponds to a final concentration of 0.32 M.

tissues. The medium shown in Table 3 was used, with certain omissions and additions. The values represent parallel measurements of oxygen uptake, net lactic acid production, and net phosphate release obtained during incubation in Warburg flasks for a period of 60 minutes at 38° C. To obtain net figures, the micromoles present in the reaction mixture at zero time (t_0) were subtracted from the values obtained after incubation.

Column 1 of Table 5 shows the results obtained with the medium precisely as in Table 3. Comparing column 1 with column 3, obtained with omission of fluoride, it is seen that fluoride stimulates oxygen uptake in all tissues except the Ehrlich ascites tumor. The effectiveness of fluoride in blocking lactic acid formation varies over a range of from 90 per cent for heart to 38 per cent for spleen. Fluoride strongly inhibits phosphate release in all tissues. No net phosphate uptake was possible even in the presence of fluoride, owing to the absence of phosphate acceptor.

The effect of antimycin A on oxygen uptake in the standard system has been described elsewhere (13, 15). In columns 5 and 6 of Table 5, fluoride has been omitted from the system, and the effect of blocking or weakening the cytochrome system by adding antimycin A or omitting cytochrome c has been studied. A comparison with column 3 shows that these procedures had little or no effect on lactic acid production. On the other hand, phosphate release was significantly reduced with heart, liver, and kidney but unaffected with spleen and the neoplastic tissues.

Fumarate addition.—All values given in Table 6

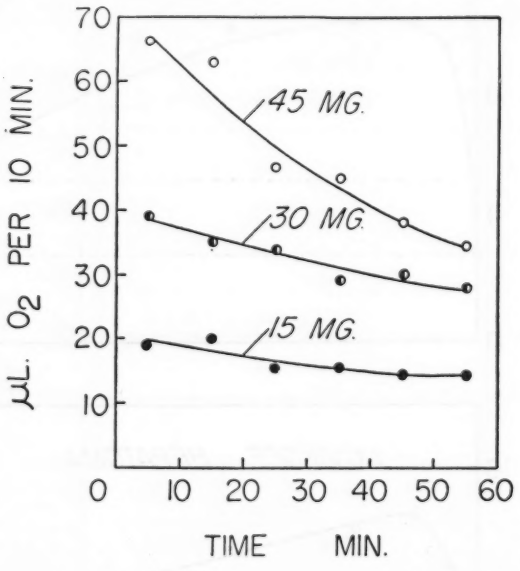

CHART 3*a*.—Variation of oxygen uptake with time for the aerobic glycolysis of HDP. A 10 per cent kidney homogenate in isotonic sucrose was used as source of enzyme.

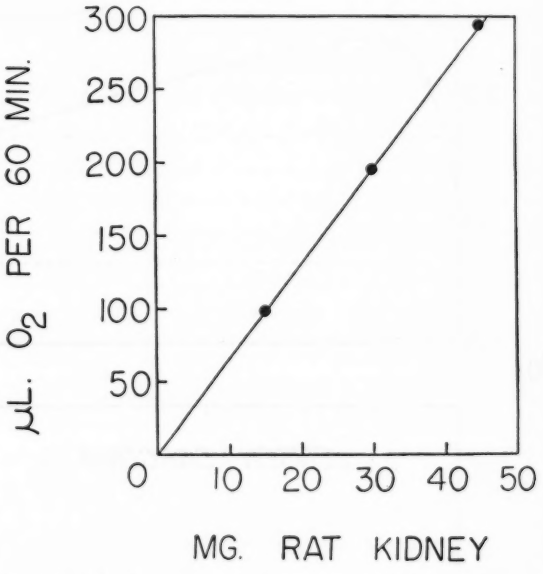

CHART 3*b*.—Dependence of oxygen uptake on tissue concentration. The data were taken from Chart 3*a*.

TABLE 4

EFFECT OF CYTOCHROME C AND BRILLIANT CRESYL BLUE (BCB) ON OXYGEN UPTAKE (Q_{O_2})* IN THE AEROBIC GLYCOLYSIS SYSTEM

	1	2	3	4	5
TISSUE	Control	Endogenous	−Cytochrome c	+BCB (0.0033 M)	−Cytochrome c +BCB
Heart	56.5	14.6	40.9	56.8	42.9
Kidney	32.8	14.3	18.0	28.2	29.4
Liver	22.4	7.8	14.0	19.8	20.0
Spleen	13.3	4.6	6.9†	13.8	12.2
Flexner-Jobling carcinoma	13.0	3.2	4.6	25.9	21.6
Jensen sarcoma	12.0	7.6	7.2	10.4	7.6
Ehrlich ascites tumor	6.8	4.2	6.8	20.8	21.3
Walker 256 carcino-sarcoma	13.3†	5.9†	5.5†	22.3†	16.7†

* Q_{O_2} values are based on oxygen uptake readings during the first 10 minutes of the experiment.

† These values were obtained in a single experiment.

TABLE 5

EFFECT OF FLUORIDE (0.01 M), ANTIMYCIN A (5×10^{-7} M), AND CYTOCHROME C (2×10^{-5} M) ON THE AEROBIC GLYCOLYSIS OF HDP (30 μM)

All measurements are for a 60-min. reaction period.

Tissue Wet wt., mg. (Dry wt., mg.)	Measurement	+KF +HDP 1	+KF Endogenous 2	+HDP 3	Endogenous 4	+Antimycin A +HDP 5	−Cyt. c +HDP 6
Heart	Q_{O_2}	38.0	6.6	24.0	10.1	7.9	18.3
20	Net lactate, μM	3.0		28.5	− 0.9	27.4	24.9
(4.5)	Net phosphate, μM	3.5	6.9†	40.7	8.9	32.9	32.7
Kidney	Q_{O_2}	22.8	10.5	21.3	9.0	15.4	18.5
30	Net lactate, μM	3.9		18.5	− 0.5	20.1	20.9
(7.1)	Net phosphate, μM	7.1†	5.9†	36.3†	8.7†	32.3†	32.3†
Liver	Q_{O_2}	18.5	7.0	16.2	7.7	15.4	12.0
30	Net lactate, μM	3.5	− 1.0	20.1	− 1.0	21.0	17.0
(9.0)	Net phosphate, μM	1.6	1.8	33.6	7.1	24.4	26.5
Spleen							
60	Q_{O_2}	4.9	2.4	3.6	2.8	3.2	3.4
(13.8)	Net lactate, μM	5.8	− 0.4†	9.3	1.2	9.8	8.9
Flexner-Jobling carcinoma	Q_{O_2}	10.7	2.6	5.4	3.8	4.8	3.1
60	Net lactate, μM	5.7	− 0.5	35.2	0.8	34.9	34.8
(9.7)	Net phosphate, μM	2.6	1.6	32.7	7.2	33.5	33.2
Jensen sarcoma	Q_{O_2}	7.2	7.0	6.0	5.3	4.8	4.0
60	Net lactate, μM	5.8		10.0	3.6	10.1	9.8
(10.6)	Net phosphate, μM	9.4	− 2.5	15.1	7.6	14.9	16.9
Ehrlich ascites tumor	Q_{O_2}	6.0	4.6	9.3	5.3	3.5	10.0
110							
(8.2)	Net lactate, μM	7.2	0.8	14.0	1.8	18.8	15.1

* Oxygen uptake is expressed as the Q_{O_2} (μl O_2/mg dry weight of tissue/hour).
† These values were obtained in a single experiment.

TABLE 6

AEROBIC GLYCOLYSIS WITH HDP (5 μM), FUMARATE (5 μM), AND GLUCOSE (30 μM) AS SUBSTRATES WITHOUT ADDITION OF FLUORIDE TO THE MEDIUM

All measurements are for a 60-min. reaction period.

Tissue Wet wt., mg. (Dry wt., mg.)	Measurement	+HDP 1	Endogenous 2	+Fumarate 3	+Fumarate +HDP 4	+Glucose 5	+Glucose +HDP 6
Heart	Q_{O_2}*	17.4	10.1			14.6†	20.2
20	Net lactate, μM	8.2	− 0.9			− 0.9†	13.2
(4.5)	Net phosphate, μM	21.1	8.9			9.8†	20.3
Kidney	Q_{O_2}	22.0	9.0	26.6†	40.0†	11.2†	23.8
30	Net lactate, μM	9.8	− 0.5	2.4†	7.3†	− 0.5†	12.8
(7.1)	Net phosphate, μM	21.6†	8.7†	10.3†	19.7†	6.8†	19.7†
Liver	Q_{O_2}	16.6†	7.7	19.1†	22.5†	7.5†	16.6†
30	Net lactate, μM	7.4	− 1.0	5.1†	12.0†	− 0.9†	7.6
(9.0)	Net phosphate, μM	23.9†	7.1	7.5†	18.5†	11.9†	23.4
Spleen	Q_{O_2}	4.0	2.8	3.5†	4.3†	2.4†	4.0
60							
(13.8)	Net lactate, μM	8.4	1.2	4.0†	8.1†	2.0†	8.9
Flexner-Jobling carcinoma	Q_O	4.4	3.8			6.0†	4.2
60	Net lactate, μM	13.3	0.8			8.3†	23.3
(9.7)	Net phosphate, μM	21.2	7.2			4.7†	20.0
Jensen sarcoma	Q_{O_2}	6.0	5.3	6.4	7.6	5.8	6.2
60	Net lactate, μM	15.0	3.6	6.7	12.7	9.1	18.0
(10.6)	Net phosphate, μM	18.8	7.6	6.9	13.6	7.0	17.8
Ehrlich ascites tumor	Q_{O_2}	8.0	5.3	11.3†	11.7†	6.4†	7.6
110							
(8.2)	Net lactate, μM	11.8	1.8	11.4†	15.0†	12.6†	16.1

* Oxygen uptake is expressed as the Q_{O_2} (μl O_2/mg dry weight of tissue/hour).
† These values were obtained in a single experiment.

were obtained with omission of fluoride from the reaction medium. Column 3 of Table 6 shows that 5 μM of fumarate stimulated oxygen uptake to a degree that was generally only slightly higher than that obtained with the same level of HDP. These results indicate that the same factors may be limiting both in Krebs cycle oxidations and in the aerobic pathway for DPNH oxidation.

A possible interpretation of the above data is that the Q_{O_2} values observed in the glycolytic system were mainly due to the oxidation of glycolysis products via the Krebs cycle. This possibility appears to be eliminated by the data of column 4, which show that the addition of both fumarate and HDP stimulated oxygen uptake beyond that found with either substrate alone in all tissues studied, but especially in kidney. This stimulation would be unlikely if HDP acted simply to raise the level of Krebs cycle substrates.

It should be noted that considerable quantities of lactic acid were produced even with fumarate as substrate. The sum of lactic acid production with HDP (column 1) and fumarate (column 3), when corrected for endogenous (column 2), was larger than the experimentally observed production of lactic acid with both HDP and fumarate present (column 4). This suggests that fumarate sparks Krebs cycle oxidation of the pyruvate that results from glycolysis.

Glucose addition.—In comparing columns 2 and 5 of Table 6, it is seen that glucose slightly stimulated the Q_{O_2} of all tissues and similarly caused only slight changes in phosphate breakdown. Of particular interest is the finding that glucose produced no increase whatever in lactic acid production with heart, kidney, and liver and only an increase of doubtful significance (0.8 μM) with spleen; on the other hand, large amounts of lactic acid were formed with all the tumor tissues tested.

These data are placed in better perspective by column 6 of Table 6, which shows that, in the case of heart and kidney, the presence of both HDP and glucose results in a larger production of lactic acid than is obtained with either substrate alone (columns 1 and 5). This result is in agreement with the previous finding in an anaerobic system (6) that a functioning glycolytic system tends to encourage lactic acid formation from glucose.

DISCUSSION

The present work confirms previous indications (10, 16) concerning the grading of DPN-cytochrome c reductase capacity from higher values in certain normal tissues to lower values in various tumor tissues, with some overlapping of data. Of particular interest is the comparison of the Q_{O_2} for the Novikoff hepatoma (Charts 1 and 2) with that of normal liver (Tables 5 and 6), which shows that the two tissues have almost the same Q_{O_2} in this system. This comparison is especially important because of the finding by Hogeboom and Schneider (2) that, in a study of this enzyme in mouse liver and the 98/15 mouse hepatoma, the latter had a significantly higher enzyme content. Lenta and Riehl (4) found almost the same activity in liver and 98/15 hepatoma but found that the hepatoma was much higher in activity than the other tumors tested.[3]

The data are also in agreement with the studies by LePage (6) on anaerobic glycolytic systems, in which it was shown that brain and tumor tissues were able to phosphorylate glucose effectively while homogenates from normal tissues other than brain were unable to glycolyze glucose.

The data for oxygen uptake in the system for aerobic glycolysis are of greatest interest in connection with the continuing discussion (cf. 19–23) as to whether tumors contain less oxidative capacity than normal tissues. If the present data are compared to those of earlier studies on the over-all malic acid system (10, 16), an interesting fact emerges—that the tumor tissues, regardless of the test system, never exceed an oxidative rate of 20–25 and are usually much lower than this, depending on the type of tumor tested. However, in the case of heart, liver, and kidney, the Q_{O_2} values in the malic system (10, 16) were 114, 103, and 81, as compared to values of 38, 18.5, and 23, respectively, in the present work. These data suggest either that the malic system was not limited by the DPN-cytochrome c reductase or that, in the aerobic glycolytic system, the triosephosphate dehydrogenase is unable to saturate the cytochrome reductase capacity in the three tissues mentioned. In the former case the complete system may have drawn in the activity of additional DPN and TPN systems and may have drawn in the succinic system as a result of the glutamate addition. What may be emphasized in any case is that the oxidative capacity of these three tissues can be shown to

[3] In evaluating these results of photometric measurements, it is relevant to consider the findings of Lehninger (3) that, when a particulate fraction of rat liver containing both mitochondria and nuclei was incubated in water for 5 minutes at 0° C. prior to the measurement of DPN-cytochrome c reductase activity, the enzyme activity was increased 4.4 times over the activity of untreated particles. This result was explained by the existence of a permeability or other structural barrier to the free diffusion of DPNH to the active centers of mitochondria. These data emphasize the fact that the physical state of the enzyme preparations used for the measurement of DPN-cytochrome c reductase activity is of great importance, and it cannot be assumed that the permeability of the mitochondria from tumors is the same as for those from liver.

be much greater than that of any of the tumor tissues studied in this laboratory or by Weinhouse and collaborators under any conditions (19–23). Among the oxidative systems that have been studied, the least ambiguous is the succinoxidase system, and again the tumors exhibit the same general level of oxidative capacity as in the other systems, while heart, liver, and kidney have capacities of 219, 88, and 195, respectively, in terms of Q_{O_2} (12). Weinhouse has apparently accepted the validity of these data but has interpreted the results as merely the reflection of the "low mitochondrial content of tumor tissues" (23). If it is agreed that the mitochondrial content of tumor tissues is low it cannot be argued that their oxidative capacity is equal to that of tissues containing vastly more mitochondria: the oxidative capacity (or mitochondrial content) of tumor cells must be judged relative to other cell components such as the nucleus or the glycolytic enzymes, or even the total cell mass. Any deficiencies in the tumor mitochondria will only accentuate the differences. While the capacities of certain individual components of the cytochrome system are still not established, it seems clear that the capacities of these components of tumors as a class are similar to those of the least active normal tissues and that the latter contain only about 1/10 as much activity as the more active normal tissues (12).

The possibility of further differences is indicated by the effects obtained with brilliant cresyl blue, which increased the oxygen uptake in three of the four tumors tested but had no effect on the normal tissues. These findings support the earlier work of Elliott and Baker (1) who observed similar effects with tissue slices. On the basis of these effects and the unexplained differences between the malic and the triosephosphate systems it is clear that further work on the individual components of the hydrogen transport system is needed. However, it is emphasized that the oxidative system of tumor tissues as judged by three types of measurement has a lower capacity than heart, liver, and kidney.

SUMMARY

1. A system for the aerobic glycolysis of HDP by tissue homogenates has been developed.

2. A grading of oxidative capacity from high values for normal tissues to low values for tumor tissues has been observed in this system. Brilliant cresyl blue doubled the oxygen uptake of three out of four tumor tissues, while no effect was observed with four normal tissues. These data are interpreted in terms of a limited capacity of the DPN-cytochrome c reductase in tumor tissues.

3. Measurements of lactic acid production and phosphate breakdown were used to study the effect of inhibitors and substrates on the aerobic glycolysis of normal and tumor tissues. Lactic acid production with HDP as substrate in the fluoride-inhibited system was similar with normal and tumor tissues. Quantitative data on the action of fluoride in stimulating oxygen uptake and inhibiting both lactic acid formation and phosphate breakdown were obtained. Addition of antimycin A or omission of cytochrome c reduced the oxygen uptake and phosphate breakdown in some tissues but had essentially no effect on lactic acid production.

4. With the probable exception of brain, which was not studied, a clear-cut difference in the capacity to produce lactic acid from glucose was observed between homogenates of normal and tumor tissues. Brain homogenates carry out this reaction anaerobically. The other normal tissues were unable to produce lactic acid from glucose in the absence of other substrates, while neoplastic tissues produced considerable quantities of lactic acid under identical conditions. However, addition of HDP stimulated the production of lactic acid from glucose in heart and kidney. These data confirm previous findings by LePage for anaerobic glycolysis.

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Effect of Thyroid Function on Tissue Metabolism of Tumor-bearing Rats*

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The most obvious abnormality of tumor tissue —that it is not controlled by the usual factors regulating normal tissue growth—raises the important question of the functioning of other controls. Since the thyroid regulates the energy metabolism of some tissues (notably liver, kidney, heart, skeletal muscle, exocrines) but not that of others (lymphoid tissue, gonads, accessory reproductive structures, brain) (1, 2) it was considered of interest to determine, first, whether hypo- or hyperthyroidism exerted any influence upon tumor oxygen consumption and, second, whether liver, kidney, and diaphragm of tumor-bearing animals would respond normally to alterations in thyroid status.

Meyer, McTiernen, and Aub (3) reported that thyroxine injected into tumor-susceptible black mice decreased the metabolic rate of Sarcoma 180 and Carcinoma 63 an average of 25 per cent, in contrast to a 20 per cent augmentation of liver oxygen consumption. Although no quantitative measurements were reported, these authors believed that the growth of Sarcoma 180 was inhibited, but not completely arrested, by the thyroxine. Thyrotrophin, desiccated thyroid, or thyroxine administered to young rats carrying Jensen sarcoma increased liver and kidney Q_{O_2} values in 2 hours, according to Reiss, Hochwald, and Druckrey (4), but the sarcoma Q_{O_2} was not altered. In view of present-day experience, it is difficult to evaluate the significance of such an early rise in metabolism.

MATERIALS AND METHODS

AXC Irish gray rats and tumor transplants were obtained through the kindness of Dr. R. J. Winzler and co-workers at the University of Southern California. The fibrosarcoma employed was originally made available by Dr. Morris K. Barrett of the National Cancer Institute.

Animals were thyroidectomized 1 or 2 days after transplantation of the tumor; no obvious differences were seen in the rate of tumor growth in unoperated and thyroidectomized animals. Three to 4 weeks later, allowing for development of hypothyroidism as well as growth of the tumor, 2 mg. of L-thyroxine[1] per kilo body weight were injected into appropriate animals each day for 4 days, and the animal was sacrificed on the 5th day. The tissues to be studied, including the tumors, were excised and immediately placed in ice-cold Krebs-Ringer solution. Liver, kidney cortex, and tumor were sliced according to the technic of Deutsch (8), great care being taken in the case of the last tissue to avoid any areas of necrosis. Oxygen constituted the gas phase in the Warburg tissue metabolism vessels. Duplicate or triplicate vessels were set up on each tissue, with Krebs-Ringer phosphate solution containing 100 mg. per cent of glucose. All results are expressed in terms of wet weight of tissue.

RESULTS AND DISCUSSION

To have as many controls as possible, littermates were divided into groups of untreated and thyroidectomized animals without and with tumor transplants and a group of thyroxine-injected thyroidectomized tumor-bearing rats. The mean oxygen consumption for each of the five types of preparations is shown in Table 1. At the beginning of the work, plans were made to take tumor biopsies for metabolic studies before commencing thyroxine injections, but this procedure led to so much necrosis and such rapid deterioration of the animals that it was abandoned. The few results obtained in this manner during the survey did not indicate any different conclusions from those reached herein.

The data reported here demonstrate clearly

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[1] The L-thyroxine was furnished by Glaxo Laboratories, Smith, Kline & French Laboratories and Baxter Laboratories.

that the presence of the tumor, rapidly growing as it was, did not alter the metabolic response of either liver or kidney to hypo- and hyperthyroidism (Table 2). Scott *et al.* (5) observed that the skin of rats with fibrosarcoma or adenocarcinoma and of mice with lymphoma, hepatoma, or mammary carcinoma took up larger quantities of I^{131} from intravenously-injected radioactive thyroglobulin than did skin of normal animals. They plants were made under the skin on the back of the animals, and it was found, when sacrificing the rats, that there had been considerable encroachment upon the thoracic cage from behind. This pressure apparently resulted in some immobilization of the diaphragm, leading to a 26 per cent diminution in its oxygen consumption. In the face of such a drastic lowering, thyroidectomy resulted in a further change of only −12 per cent. How-

TABLE 1

OXYGEN CONSUMPTION OF TISSUES OF AXC IRISH GRAY RATS

ANIMAL*	Oxygen consumption (mm^3 O /mg/hr ± S.D.†)			
	Tumor	Liver	Kidney	Diaphragm
Normal (8)		1.44±0.04†	3.66±0.16	0.91±0.07
Normal (6)	0.78±0.04	1.39±0.10	3.79±0.20	0.67±0.08
Thyroidectomized (6)		1.02±0.10	3.27±0.16	0.67±0.12
Thyroidectomized (7)	0.77±0.12	0.99±0.18	3.21±0.11	0.59±0.05
Thyroxine-injected thyroidectomized (6)	0.74±0.13	1.56±0.09	4.35±0.28	0.92±0.06

* The number of animals in each group is shown in parentheses.

† Standard deviation = $\sqrt{\frac{\Sigma d^2}{n-1}}$.

TABLE 2

EFFECTS OF THYROIDECTOMY (TX) AND OF THYROXINE INJECTION ON METABOLISM OF TISSUES OF AXC IRISH GRAY RATS

COMPARISON	Tissues studied							
	Tumor		Liver		Kidney		Diaphragm	
	Change (per cent)	P	Change (per cent)	P	Change (per cent)	P	Change (per cent)	P
Tumor Control / Control			− 3.5	>0.10	+ 3.6	>0.10	−26.4	<0.01
Tx / Control			−29.2	<0.01	−10.7	<0.01	−26.4	<0.01
Tumor Tx / Tumor Control	−1.3	>0.10	−28.8	<0.01	−15.3	<0.01	−11.9	<0.10 >0.05
Tumor Tx+Thyroxine / Tumor Tx	−3.9	>0.10	+57.6	<0.01	+35.5	<0.01	+55.9	<0.01
Tx* / Control			−19.8	<0.01	−14.8	<0.01	−30.6	<0.01
Tx+Thyroxine* / Tx			+62.4	<0.01	+47.6	<0.01	+72.7	<0.01

* Values obtained by Barker and Schwartz (2).

considered this as a "reflection of disturbed metabolism referable to a relationship between tumor and host." However, liver and kidney uptakes were normal or slightly lowered; muscle, gastrointestinal tract, and blood were normal or slightly increased. Whatever the cause or significance of the elevated skin I^{131} uptake, there is no evidence that the basic metabolic potentialities of either liver or kidney are altered by the presence of a tumor outside of these organs.

In the case of diaphragm, the Q_{O_2} was lowered in the animals carrying tumors. The tumor transever, since the tissue could still respond to thyroxine injection, it seems justifiable to conclude that no qualitative change in metabolic activity was brought about by the tumor.

It will be noted from the results shown in the tables that the metabolic rate of the fibrosarcoma did not vary significantly with the thyroid status. This agrees with the findings reported with Jensen sarcoma (4) but is in some contrast to the 25 per cent average decrease in oxygen consumption claimed by Meyer *et al.* (3) for Sarcoma 180 and Carcinoma 63 carried by tumor-susceptible black

mice. These authors also recorded their impression of a growth inhibition caused by thyroxine injection, implying a more widespread depression of tumor functioning than was apparent from our experiments. Gross observation showed no less rapid growth of the fibrosarcomas in our hypothyroid animals than in the euthyroid, and no clear differentiation even between the hyperthyroid and the thyroidectomized. In all types of animals, tumor implants allowed to grow more than 5 weeks became so large that the animals could move in their cages only with great difficulty. Smith and co-workers (6) have recently reported that injections of somatotrophin caused an increased growth of mammary adenocarcinoma in C3H mice. Likewise, adrenalectomy or hypophysectomy in rats (tube-fed to insure adequate intake) inhibited the growth of intramuscular Walker carcinosarcoma 256 (7). Since neither our study nor that of Meyer *et al.* was conducted in such a manner as to enable truly quantitative measurement of tumor growth, conclusions concerning the effects of hypo- and hyperthyroidism on growth must remain less definite.

Sections were made of tumors obtained from animals at all levels of thyroid function,[2] but careful cytological study revealed no differences, in keeping with the absence of growth or metabolic differences. Fibrosarcoma tissue is so different from any of the types of tissues shown to respond to thyroid control that it perhaps is not surprising to find that it is insensitive. Other types of tumors, such as hepatomas and adenomas, should be tried.

SUMMARY

Fibrosarcoma tissue grown in AXC Irish gray rats has been found not to respond metabolically to hypo- or hyperthyroidism, although it had no influence upon the changes in oxygen consumption of liver or kidney typical of these conditions. Physical encroachment of the tumor bulk upon the thorax apparently was responsible for lowering the metabolic activity of diaphragm. However this tissue still responded to hypo- and hyperthyroidism. There appeared to be no marked changes in growth rate or cytological appearance of the tumors in the different states of thyroid function.

[2] Appreciation is expressed to Miss Alice Rudolph, Department of Zoology, University of California at Los Angeles, for her work preparing these sections.

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Announcements

Notice Regarding VIth International Cancer Congress

Physicians and scientists are invited to present papers at the VIth International Cancer Congress to be held in São Paulo, Brazil, on July 23–29, 1954. The Congress is sponsored by the International Union against Cancer. The program will include sections on fundamental cancer research, on clinical studies on cancer, and on cancer control.

In accordance with similar arrangements being made in other countries to co-ordinate participation in the Congress, residents of the United States who desire to present papers must send *five* copies of an abstract of each paper to be presented to the Chairman, National Committee on the International Union against Cancer, National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D.C., *before 15 January, 1954*. Abstracts are not to exceed 250 words and must be accompanied by a title and the name, address, academic or professional title, and institutional affiliation of the investigator or physician. These requirements do not apply to people who have been invited to participate by the President of the Congress unless application is made for travel allotments as described below.

Travel allotments of approximately $600 each will be available to a limited number of individuals requiring such assistance. Applications for travel allotments must be submitted in quintuplet to the above address *by 15 January, 1954*. They should be in letter form giving information concerning age, training, publications in cancer or related fields, and academic or professional status. Applicants not planning to present papers should include five copies of abstracts, as described above, of major current investigative work. A letter from the laboratory director, or appropriate administrative officer, approving the application is also necessary. It is expected that round-trip transportation by air from Miami to São Paulo will be available for approximately $500.

Registration blanks and hotel reservation blanks are available at the above address.